PROPIONATES

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7. Comparison of specific radioactivities of blood lactate and plasma glucose during the infusion of [^{14}C]glucose or [^{14}C]lactate indicated that about 6% of glucose was recycled through the glucose–lactate interconversion. Evidence from the relocation of ^{14}C in glucose during the infusion of [6-^{14}C]glucose indicated about 10% of recycling.

8. Negligible incorporation of glucose or lactate into liver or muscle glycogen was observed.

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The Metabolism of Acetic Acid, Propionic Acid and Butyric Acid in Sheep

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The production in the rumen of large amounts of acetic acid, propionic acid and butyric acid, which are absorbed into the portal system, is well established. Propionate and butyrate are largely removed by the liver under normal feeding conditions, but substantial quantities of acetate appear in peripheral blood (Annison, Hill & Lewis, 1957). The possible glucogenicity of acetate and butyrate in ruminants was studied previously with liver slices (Leng & Annison, 1963), when no evidence was obtained of net synthesis of carbohydrate from these substrates. These results have been confirmed in the present studies with intact animals. Solutions of [^{14}C]acetate, [^{14}C]propionate or [^{14}C]butyrate were infused into the portal vein of anaesthetized sheep, and the incorporation of ^{14}C into blood glucose, lactate and ketone bodies, and into liver and muscle glycogen, was examined. Some acetate production from propionate and butyrate was demonstrated.

MATERIALS AND METHODS

Experimental animals. Merino wethers (2–3 years of age) were housed indoors and fed once daily (early morning) on a standard ration of 800 g. of lucerne chaff. Animals were not fed on the day of an experiment.

Experimental procedure. Polyethylene catheters were inserted into both jugular veins on the day before an experiment. Solutions of the sodium salts of [^{14}C]acetic acid, [^{14}C]propionic acid or [^{14}C]butyric acid were infused at the rate of 0·1 μc/min./kg. body wt. for 60 min. into the portal vein of anaesthetized animals by the procedure described by Annison & Lindsay (1962). Negligible amounts of unlabelled sodium acetate (0·5 μmole/min./kg.) were infused with [1-^{14}C]acetate and [2-^{14}C]acetate, but with [1-^{14}C]-propionate, [2-^{14}C]propionate, [1-^{14}C]butyrate, [2-^{14}C]-butyrate and [3-^{14}C]butyrate, unlabelled substrate was infused at a rate of 5 m-moles/min./kg. Blood samples were withdrawn from the right jugular vein 60 min. after the start of the infusion. At the end of the infusion samples of liver and muscle were removed as quickly as possible for assay of glycogen.

^{14}C-labelled fatty acids. The ^{14}C-labelled and unlabelled fatty acids used in the present studies were as described by Leng & Annison (1963).

Organism. A culture of *Leuconostoc mesenteroides* (strain 39) was obtained from the Department of Bacteriology, Indiana University, Bloomington, Ind., U.S.A.

Chemical methods. The methods used for the measurement of plasma glucose concentration and specific radioactivity were as described by Annison & White (1961). Glucose was isolated from blood as described by Annison & White (1962*a*), and the distribution of ^{14}C determined by stepwise degradation with *L. mesenteroides* (Leng & Annison, 1963). The measurement of blood ketone bodies, the isolation of β-hydroxybutyrate by column chromatography and its stepwise degradation were as described by Leng & Annison (1963). Formic acid was measured by gas chromatography, and assayed for radioactivity as described by Annison & White (1962*a*). Acetic acid, propionic acid and butyric acid were isolated from blood by gas–liquid chromatography and their specific radioactivities determined by the methods described by Annison & Lindsay (1962). Acetic acid was degraded by a modification of the method of Phares (1951) as used in the degradation of glucose (Leng & Annison, 1963). Lactic acid concentrations and specific radioactivities were determined as described by Annison, Lindsay & White (1963), the acid being isolated from blood as the zinc salt, and on some occasions purified by column chromatography (Leng & Annison, 1963). The distribution of ^{14}C in lactic acid was determined as described by Leng & Annison (1963).

RESULTS

Incorporation of fatty acids into glycogen, glucose and lactate. The incorporation of ^{14}C into glucose and glycogen from [^{14}C]propionate was greater than that from [^{14}C]acetate or [^{14}C]butyrate, but the degree of labelling of glycogen in each case was low relative to that of plasma glucose (Table 1). The specific radioactivity of lactate was lower than that of glucose after the infusion of all ^{14}C-labelled fatty acids, except [2-^{14}C]butyrate when it slightly exceeded that of glucose.

The distribution of ^{14}C in plasma glucose and blood lactate after the infusion of ^{14}C-labelled fatty acids is shown in Table 2. The results confirm the glucogenicity of propionate, but the labelling patterns in glucose and lactate from [^{14}C]acetate and [^{14}C]butyrate were consistent with entry through acetyl-CoA and the tricarboxylic acid cycle (Weinman, Strisower & Chaikoff, 1957). The labelling patterns in glucose obtained from [1-^{14}C]-acetate, [1-^{14}C]butyrate or [3-^{14}C]butyrate were closely similar. The pattern from [2-^{14}C]acetate was similar to that from [2-^{14}C]butyrate.

Incorporation of fatty acids into β-hydroxybutyrate, acetate and formate. Infusions of [^{14}C]butyrate and [^{14}C]acetate gave rise to ^{14}C-labelled blood β-hydroxybutyrate, the raised concentrations of β-hydroxybutyrate observed during infusions of butyrate probably occurring in response to the infusion of carrier butyrate (Table 1). Partial degradation of ^{14}C-labelled β-hydroxybutyrate isolated from blood after the infusion of [1-^{14}C]-, [2-^{14}C]- or [3-^{14}C]-butyrate indicated that 86, 0·1 and 8 % respectively of the total ^{14}C was located at C-1. The production of ^{14}C-labelled β-hydroxybutyrate from [1-^{14}C]- or [2-^{14}C]-propionate was unexpected, but consistent with the appearance of

Table 1. *Incorporation of ^{14}C from [^{14}C]acetate, [^{14}C]propionate and [^{14}C]butyrate into glycogen and blood constituents in sheep*

The specific radioactivities and concentrations of blood acetate, propionate, butyrate, lactate and β-hydroxybutyrate, and of plasma glucose, were measured 60 min. after the start of infusions of labelled fatty acids. Experimental details are given in the text.

	Concentration (μmoles/ml.)				
Fatty acid infused	Fatty acid infused	Acetate	β-Hydroxybutyrate	Glucose	Lactate
[1-^{14}C]Acetate	0·59	0·59	0·20	4·20	0·90
[2-^{14}C]Acetate	0·52	0·52	0·13	4·10	1·31
[1-^{14}C]Propionate	0·40	0·21	0·08	4·35	0·99
[2-^{14}C]Propionate	0·35	0·25	0·23	3·55	0·80
[1-^{14}C]Butyrate	0·12	1·02	0·38	4·35	1·07
[2-^{14}C]Butyrate	0·15	0·83	0·82	7·20	1·63
[3-^{14}C]Butyrate	0·13	0·58	0·84	2·70	0·99

	Specific radioactivity (μc/g. of C)						
Fatty acid infused	Fatty acid infused	Acetate	β-Hydroxybutyrate	Glucose	Lactate	Liver glycogen	Muscle glycogen
[1-^{14}C]Acetate	72	72	4·7	2·0	1·0	0·008	0·006
[2-^{14}C]Acetate	92	92	14·0	5·1	0·6	0·006	0·001
[1-^{14}C]Propionate	—	0·4	1·8	13·6	3·5	0·04	0·001
[2-^{14}C]Propionate	123	0·4	2·6	37·9	5·3	0·22	—
[1-^{14}C]Butyrate	103	6·4	45·5	3·4	1·8	0·05	0·005
[2-^{14}C]Butyrate	166	13·2	51·2	4·2	4·8	0·01	0·002
[3-^{14}C]Butyrate	174	17·2	59·2	2·8	2·1	0·01	0·008

Table 2. *Distribution of ^{14}C in glucose and lactate isolated from plasma in sheep after the infusion of specifically labelled [^{14}C]acetate, [^{14}C]propionate or [^{14}C]butyrate*

The rates of infusion were: [^{14}C]acetate, 0·5 μmole/min./kg. body wt. (0·1 μc/min./kg.); [^{14}C]propionate and [^{14}C]butyrate, 5 m-moles/min./kg. body wt. (0·1 μc/min./kg.). The period of infusion was 60 min. in all cases. Experimental details are given in the text.

	Distribution of ^{14}C in glucose and lactate carbon atoms (%)								
	Glucose						Lactate		
Fatty acid infused	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3
[1-^{14}C]Acetate	3	7	40	46	3	1	88	8	6
[2-^{14}C]Acetate	23	22	9	9	26	11	26	48	26
[1-^{14}C]Propionate	1	2	47	45	4	1	84	11	5
[2-^{14}C]Propionate	25	20	4	4	22	25	10	50	40
[1-^{14}C]Butyrate	2	4	38	45	8	3	79	8	13
[2-^{14}C]Butyrate	26	16	8	10	22	18	25	37	38
[3-^{14}C]Butyrate	5	6	41	39	4	5	85	6	9

^{14}C-labelled blood acetate during the infusion of [1-^{14}C]- or [2-^{14}C]-propionate. Stepwise degradation of the ^{14}C-labelled acetate arising during the infusion of [1-^{14}C]- or [2-^{14}C]-propionate revealed that in each case the ^{14}C was distributed approximately equally between C-1 and C-2.

^{14}C-labelled formate was detected in blood after the infusion of [^{14}C]acetate, [^{14}C]propionate or [^{14}C]butyrate. Considerable variation between experiments was observed, the specific radioactivity of formate isolated from blood ranging from 1 to 5 % of that of the ^{14}C-labelled fatty acid.

The infusion of sodium propionate (5 m-moles/min./kg.) invariably depressed blood acetate concentrations (Table 1).

DISCUSSION

The evidence for the possible glucogenicity of butyrate and acetate was reviewed by Leng & Annison (1963). The results of the present studies confirmed the previous findings, with liver slices, that the incorporation of acetate C and butyrate C into glucose in sheep tissues involves no net synthesis of carbohydrate (Leng & Annison, 1963). Kronfeld, Campbell, Hooper & Galligan (1959) have suggested that butyrate may be incorporated into glucose as a C_3 unit (acetone) via the propanediol pathway. Conversion of butyrate into acetoacetate undoubtedly gives rise to acetone which may be directly converted into a glycolytic intermediate in some animal tissue (Mourkides, Hobbs & Koeppe, 1959; Shull & Miller, 1960). However, the high specific radioactivity of blood β-hydroxybutyrate, and the relatively little ^{14}C at C-2 and C-5 of glucose isolated after the infusion of [3-^{14}C]butyrate, indicate that pathways involving acetone are not quantitatively important in butyrate metabolism in sheep tissues. Similar conclusions were reached by Black, Kleiber & Brown (1961), in studies with the lactating cow.

The incorporation of ^{14}C from [1-^{14}C]- or [2-^{14}C]-propionate into glucose was consistent with the conversion of propionate into a symmetrical C_4 compound before conversion into phosphoenolpyruvate, and was in agreement with the established pathway, namely propionate → methylmalonate → succinate → phosphoenolpyruvate → glucose. The equal distribution of ^{14}C between C-2 and C-3 of lactate arising from [2-^{14}C]propionate was evidence against metabolic pathways involving the direct conversion of propionate into lactate (or pyruvate), e.g. propionate → acrylate → lactate → pyruvate (Mahler & Huennekens, 1953). The slight incorporation of ^{14}C into acetate and β-hydroxybutyrate from [1-^{14}C]propionate implies the production of acetyl-CoA from propionate by a pathway other than the well-established route of propionate → succinate → pyruvate → acetyl-CoA, which would result in loss of ^{14}C as $^{14}CO_2$. Comparison of the specific radioactivities of blood propionate, acetate and β-hydroxybutyrate (Table 1) indicated that this pathway was not quantitatively significant. The high specific radioactivity of β-hydroxybutyrate relative to that of acetate probably reflected the greater rate of acetate entry into body pools. James, Peeters & Lauryssens (1956) reported the production of ^{14}C-labelled acetate from [1-^{14}C]propionate by the perfused goat udder.

Comparison of the specific radioactivities of blood β-hydroxybutyrate and blood fatty acid after the infusion of [^{14}C]acetate and [^{14}C]butyrate (Table 1) indicated the high incorporation of butyrate C relative to that of acetate C into β-hydroxybutyrate. In non-ruminants the production of ketone bodies is largely based on condensation of acetyl-CoA (Krebs, 1961), but the labelling

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urate

.); [^{14}C]propionate and
was 60 min. in all cases.

atoms (%)

Lactate	
C-2	C-3
8	6
48	26
11	5
50	40
8	13
37	38
6	9

Black, Kleiber & Brown
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patterns in β-hydroxybutyrate produced from [^{14}C]butyrate indicated the conversion of butyrate into β-hydroxybutyrate largely as a C_4 unit, emphasizing the special role of butyrate in ketone body metabolism in ruminants. The low contribution of acetate to β-hydroxybutyrate production probably reflects low utilization of acetate by liver tissues, a major site of ketone body production.

The isolation of lactate of slightly higher specific radioactivity than that of glucose after the infusion of [2-^{14}C]- but not of [1-^{14}C]- or of [3-^{14}C]-butyrate was unexpected, and inconsistent with the evidence from glucose and lactate labelling patterns that butyrate was metabolized as two C_2 units. The possible contamination of lactate with β-hydroxybutyrate cannot be excluded, although control experiments showed that inactive zinc lactate could be recovered from blood (10 ml.) containing added lithium lactate (50 mg.) and β-hydroxybutyrate (10 mg., 10 μmc).

The infusion of [2-^{14}C]acetate or [2-^{14}C]butyrate resulted in appreciable labelling of C-3 and C-4 of glucose. In experiments in which these ^{14}C-labelled fatty acids were incubated with liver slices (Leng & Annison, 1963) much less ^{14}C was found in C-3 and C-4 of glucose. Propionate metabolism was shown by Leng & Annison (1963) to lead to increased fixation of CO_2 arising from butyrate oxidation. Therefore, the increased labelling of C-3 and C-4 of glucose in the experiments with the whole animal may reflect greater availability of propionate.

The relatively low specific radioactivity of formate produced from [^{14}C]acetate, [^{14}C]propionate and [^{14}C]butyrate suggested that these short-chain fatty acids are not major precursors of formate.

The concentration of acetate in blood was markedly depressed by the infusion of propionate (Table 1). Concentrations in starved sheep with rumen contents removed are rarely lower than 0·3 μmole/ml. (Annison & White, 1962*b*), and the present results suggest that propionate either inhibits endogenous acetate production or increases rates of removal of acetate from blood.

The results of the present studies suggest that, in spite of the dependence of ruminants on large amounts of short-chain fatty acids produced by fermentation in the rumen, the metabolic pathways involved in their utilization are similar to those known to occur in non-ruminants (cf. Lifson, Lorber, Sakami & Wood, 1948).

SUMMARY

1. Portal infusions of [1-^{14}C]acetate, [2-^{14}C]acetate, [1-^{14}C]propionate, [2-^{14}C]propionate, [1-^{14}C]-butyrate, [2-^{14}C]butyrate or [3-^{14}C]butyrate were made into anaesthetized sheep.

2. The specific radioactivity of liver and muscle glycogen was low after all infusions. The highest value was less than 0·2 % of the specific radioactivity of the fatty acid infused ([2-^{14}C]propionate).

3. The specific radioactivity of plasma glucose was 2–5 % that of the fatty acid infused after the infusion of acetate or butyrate, but 30 % after the infusion of [2-^{14}C]propionate.

4. The specific radioactivity of blood lactate was less than that of blood glucose except after the infusion of [2-^{14}C]butyrate, when it was very slightly higher.

5. The infusion of [1-^{14}C]acetate, [1-^{14}C]propionate, [1-^{14}C]butyrate or [3-^{14}C]butyrate gave rise to labelling mainly in C-3 and C-4 of glucose and C-1 of lactate. The infusion of [2-^{14}C]acetate, [2-^{14}C]propionate or [2-^{14}C]butyrate labelled mainly C-1, C-2, C-5 and C-6 of glucose, and C-2 and C-3 of lactate.

6. The specific radioactivity of blood β-hydroxybutyrate was about 50 % of that of infused [^{14}C]-butyrate, about 7–15 % of infused [^{14}C]acetate and about 2 % of infused [^{14}C]propionate.

7. The specific radioactivity of blood formate was about 5 % of that of infused [^{14}C]acetate, [^{14}C]propionate or [^{14}C]butyrate.

8. The specific radioactivity of blood acetate was less than 1 % of that of infused [^{14}C]propionate but about 6–10 % of that of infused [^{14}C]butyrate.

9. The results are consistent with the operation of metabolic pathways similar to those known to occur in non-ruminants.

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TRANSFER OF SHORT-CHAIN FATTY ACIDS BY THE INTESTINE

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Smyth & Taylor (1958) have shown that acetic, propionic and butyric acids can be transferred against a concentration gradient by preparations of rat intestine *in vitro*. These studies were limited to observations on the concentration of fatty acid in mucosal and serosal fluids and to measurement of changes in the amount of fatty acid and water on the serosal side. The present work extends these observations to further details of movement of these fatty acids and to higher members of the fatty acid series. The results also provide a basis for discussing the interpretation of intestinal transfer studies *in vitro*.

METHODS

In vitro. The preparation was the sac of everted small intestine of the rat (Wilson & Wiseman, 1954). The sacs were 30 cm long, made from the jejunum of the rat, contained 3 ml. bicarbonate saline (Krebs & Henseleit, 1932) with 500–1000 mg glucose/100 ml., and were suspended in 15–50 ml. of the same solution. They were shaken at a rate of 80 c/min for 60 min at 38° C in conical flasks, as described by Parsons, Smyth & Taylor (1958). Two types of experiments were done as also described by these authors: (1) transfer experiments, in which the object of the experiment was to measure the changes in the mucosal and serosal fluids; and (2) metabolism experiments, where the object was to estimate the total amount of fatty acid disappearing during the course of the experiment. In the metabolism experiments, the method was modified as follows. After incubation of the sac for 1 hr the flask was immersed in an ice-salt freezing mixture (approximately −10° C) until the contents froze (approximately 3 min). Cold water was then run over the outside of the flask until the contents just thawed. The sac was removed and ground with 1 ml. of acid-washed sand, 5 ml. 7 % (w/v) $CuSO_4.5H_2O$ and 5 ml. 10 % (w/v) $Na_2WO_4.2H_2O$. The ground-up tissue together with the deproteinizing reagents was pooled with the flask contents, and the total volume made up to 100 ml. After filtering, samples were taken for estimation of volatile fatty acid.

Expression of results. The terms used in expressing the results are as defined by Parsons *et al.* (1958), and for convenience they are repeated here. 'Mucosal fluid transfer' is the decrease in volume of the mucosal fluid; 'serosal fluid transfer' is the increase in volume of the empty sac; 'mucosal of the serosal fluid; 'gut fluid uptake' is the increase in volume of the empty sac; 'mucosal substrate transfer' is the decrease in amount of substrate (in this case fatty acid) on the mucosal side; 'serosal substrate transfer' is the increase in amount of substrate on the serosal side. 'Final concentration gradient' is the difference between the final serosal substrate concentration and final mucosal substrate concentration. In addition to the above

terms the following are also used: 'gut substrate uptake' is the amount of substrate not accounted for in the final mucosal and serosal fluids and may be due either to metabolism or to accumulation of substrate in the intestine. 'Mucosal concentration transferred' is the mucosal substrate transfer divided by the mucosal fluid transfer, and the 'serosal concentration transferred' is the serosal substrate transfer divided by the serosal fluid transfer. If the figure of 3·2 for the wet wt.:dry wt. ratio found by Fisher & Parsons (1953*a*) is accepted, the 'initial gut fluid content' can be calculated from the initial wet weight, and if to this is added the 'gut fluid uptake', the 'final gut fluid content' is obtained. If the amount of substrate in the gut at the end of the experiment is known the 'final gut substrate concentration' can be calculated. The above quantities as defined are considered as positive. Under certain conditions some of them may become negative. In comparing the results of different experiments it is usually convenient to express the transfer per gram initial wet weight.

When many experiments are done, the calculation of all the above quantities becomes laborious. The work has been greatly facilitated by the use of the Pegasus Electronic Computer. A programme for the work was prepared by Mr P. H. Blundell, and this very greatly reduced the time spent on calculation.

In vivo. The method for studying absorption *in vivo* was that of Sheff & Smyth (1955), as modified by Jervis, Johnson, Sheff & Smyth (1956). Incubation of a sample of mucosal fluid containing glucose did not show any production of volatile fatty acid, thus excluding possible formation of volatile fatty acid by bacterial action.

Chemical. The fatty acids were obtained commercially and were used as sodium salts. They were estimated by steam distillation in a Markham still after deproteinization with 7 % (w/v) $CuSO_4.5H_2O$ and 10 % (w/v) $Na_2WO_4.2H_2O$. The final titration was with 0·05 N-NaOH, with phenol red as indicator.

Statistical. In the section on Michaelis-Menten kinetics the statistical procedures used were those described by Davies & Jowett (1959).

RESULTS

Metabolism of fatty acids

The purpose of these experiments was to determine the capacity of the intestine to metabolize or produce fatty acid. The production of volatile fatty acid in the absence of added fatty acid is given in Table 1, which includes experiments in the presence and absence of glucose, and experiments where the sac was suspended in saline containing glucose but removed without having been incubated. Two experiments were also done in which lengths of intestine were extracted immediately after removal without making into a sac. These results show that incubation of the sac produces a small amount of volatile fatty acid which is possibly slightly greater in the presence of glucose. Without incubation the amount of volatile fatty acid produced is insignificant. Table 2 shows the relation between the amount of fatty acid added and the amount recovered in the presence and absence of glucose for acetate, propionate and butyrate, respectively. In the case of acetate and butyrate, fatty acid disappeared during incubation in the absence of glucose, and this disappearance is more striking if the amount of fatty acid produced in the absence of added

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TABLE 1. Production of volatile fatty acid by 30 cm sacs of everted small intestine when no fatty acid was present initially. The sacs were suspended in Krebs's bicarbonate saline, with or without added glucose. In the last two experiments shown, 30 cm of intestine was ground up immediately after removal from the animal without eversion

Glucose concn. (mg/100 ml.)	Time of incubation (min.)	Volatile fatty acid (μmole)	Mean (μmole)
500	60	7·7	7·7
500	60	9·5	
500	60	6·8	
500	60	6·8	
0	60	4·7	5·4
0	60	6·0	
500	0	5·7	2·8
500	0	3·1	
500	0	1·4	
500	0	0·9	
0	0	2·4	2·6
0	0	2·8	

TABLE 2. Change in total amount of volatile fatty acid after 60 min incubation of sacs of everted small intestine in Krebs's bicarbonate saline with fatty acid added initially in the presence or absence of glucose. The initial concn. of fatty acid was approximately 10 mM in all cases. In the column marked 'difference' a positive quantity indicates production of volatile fatty acid and a negative quantity disappearance

Fatty acid	Initial glucose concn. (mg/100 ml.)	Volatile fatty acid (μmole) Initial	Final	Difference	Mean
Acetate	1000	178	178	0	− 1
	1000	178	175	− 3	
	1000	179	177	− 2	
	1000	179	180	1	
	0	179	168	−11	−13
	0	179	166	−13	
	0	180	164	−16	
	0	180	168	−12	
Propionate	1000	178	183	5	5
	1000	178	182	4	
	1000	161	167	6	
	1000	161	167	6	
	0	161	163	2	3
	0	161	162	1	
	0	178	183	5	
	0	178	182	4	
Butyrate	1000	175	172	− 3	− 5
	1000	175	172	− 3	
	1000	182	178	− 4	
	1000	182	176	− 6	
	1000	182	174	− 8	
	0	175	163	−12	−11
	0	175	167	− 8	
	0	182	169	−13	
	0	182	172	−10	
	0	182	168	−14	

fatty acid is also considered. In contrast there appeared to be almost no metabolism of propionate.

TABLE 3. Transfer of acetate and fluid by everted sac of rat jejunum. The initial wet weight of the sac was 1·98 g. Duration of experiment 60 min

Acetate (μmole)	
Mucosal transfer	161
Serosal transfer	72
Gut uptake	89
Fluid (ml.)	
Mucosal transfer	2·58
Serosal transfer	1·56
Gut uptake	1·02
Acetate concn. (mM)	
Initial mucosal and serosal	20·3
Final mucosal	11·5
Final serosal	29·2
Final concn. gradient	17·7
Mucosal concn. transferred	62·4
Serosal concn. transferred	46·2

Transfer of fatty acids

The general pattern can be seen by reference to one experiment with acetate (Table 3). The initial concentration was the same in mucosal and serosal solutions, i.e. 20·3 mM. During the experiment 161 μmole acetate and 2·58 ml. fluid left the mucosal side. The acetate concentration in the fluid leaving the mucosal side (mucosal concentration transferred) was therefore 62·4 mM i.e. about three times the initial concentration in the mucosal fluid. Of the acetate and fluid leaving the mucosal side, some was retained in the gut and some was transferred to the serosal side. The gut acetate uptake (89 μmole) was proportionately greater than the gut fluid uptake (1·02 ml.) with the result that the serosal concentration transferred (46·2 mM) was less than the mucosal concentration transferred. The serosal concentration transferred was, however, greater than the initial concentration of acetate on the serosal side, with the result that the final serosal concentration of acetate (29·2 mM) was greater than the initial serosal concentration (20·3 mM). Of the 89 μmole of acetate taken up by the tissue, presumably a small amount was metabolized and the rest retained in the gut wall. Similar results were obtained in more than fifty other experiments with acetate. Analogous results were also obtained with propionate, butyrate, *n*-valerate, *iso*-valerate and *n*-hexanoate.

Significance of serosal transfer

As a considerable amount of water and fatty acid is present in the gut wall at the end of the experiment, the relation was determined between the concentration of fatty acid in the gut wall, the final serosal concentration, the mucosal concentration transferred and the serosal concentration

transferred. Since propionate is not metabolized it was used for these experiments, Table 4 gives the results of one complete experiment for propionate, in which the amount in the gut wall was determined by two methods; (*a*) by difference between the mucosal transfer and the serosal transfer and (*b*) by direct determination of the amount in the gut wall at

TABLE 4. Transfer of propionate and fluid by everted sac of rat jejunum. The initial weight of the sac was 1·94 g. Duration of experiment 60 min

Propionate (μmole)	
Mucosal transfer	92
Serosal transfer	54
Gut uptake as calculated	38
Gut uptake as estimated directly	42
Fluid (ml.)	
Mucosal transfer	3·01
Serosal transfer	2·34
Gut uptake	0·67
Initial gut fluid content	1·29
Final gut fluid content	1·96
Propionate concn. (mM)	
Initial mucosal and serosal	10·0
Final mucosal	7·2
Final serosal	15·8
Final concn. gradient	8·6
Mucosal concn. transferred	30·6
Serosal concn. transferred	23·1
Final gut propionate concn.:	
from gut uptake as calculated	19·4
from gut uptake estimated directly	21·4

TABLE 5. Comparison of final gut propionate concn. with the serosal concn. transferred and the final serosal concn. The initial mucosal and serosal concn. were the same in each experiment. The figures given are average values for the number of experiments shown. Duration of experiments 60 min

Initial propionate concn. (mM)	Number of experiments	Mucosal concn. transferred (mM)	Serosal concn. transferred (mM)	Final gut concn. (mM)	Final serosal concn. (mM)
5	6	17·4	15·1	9·4	9·2
10	6	31·5	26·3	18·7	17·2
20	7	57·9	43·2	35·6	28·9
46	3	99·5	68·1	56·6	53·4

the end of the experiment. Table 5 shows results obtained from a number of experiments, in which the amount of propionate in the gut wall was calculated from the difference between mucosal and serosal transfer. From these results it is seen that (1) there is good agreement between the values obtained for gut uptake as calculated and as obtained by direct determination; (2) the mucosal concentration transferred was always greater than the serosal concentration transferred, and (3) the final gut concentration was always intermediate between the serosal concentration

transferred and the final serosal concentration. The following interpretation of these results is offered. The fluid and solutes which are transferred by the epithelial cells first pass into the subepithelial tissues. When these can hold no more fluid, the excess spills over into the sac contents. Since the gut wall contains initially fluid but no fatty acid, the propionate concentration in the gut wall must be less than the mucosal concentration transferred, although it will presumably increase during the course of the experiment. If the serosal transfer was only a spill over of fluid from the gut wall, the concentration in the fluid being transferred at any instant could never exceed the concentration in the gut wall, and the average serosal concentration transferred, i.e. what is measured, would probably be less than and could not be greater than the final gut concentration. In fact the final gut concentration was found to be less than the serosal concentration transferred, and this shows that in addition to the spill over there is diffusion of propionate from the higher concentration in the gut wall to the lower concentration inside the sac, but there has been insufficient time to achieve equilibrium. The importance of this interpretation is to stress that the serosal transfer represents only one fraction of what is transferred by the epithelial cells, the rest being in the gut wall. It therefore seems preferable to take mucosal transfer as a measure of transfer capacity rather than serosal transfer.

Effect of variations in fatty acid concentration

The transfer of acetate was studied in concentrations from 5 to 80 mM and the results are seen in Figs. 1–3. Figure 1 shows the mucosal transfer of acetate and water. Acetate transfer increases with increase in initial concentration up to 60 mM, and beyond this point there is reduction in mucosal transfer. In contrast, water transfer remains fairly constant up to a concentration of 40 mM, but at 60 mM there is some reduction in transfer, and at 80 mM a very considerable reduction. Higher concentrations of acetate evidently have an inhibitory effect on transfer of both acetate and fluid. The relation between water transfer and acetate transfer is not, however, direct and at least some independent variation of acetate and water transfer is possible.

Figure 2 shows the mucosal and serosal acetate concentrations transferred, i.e. the concentration of acetate in the fluid leaving the mucosal solution and the concentration in the fluid entering the serosal side. The straight line in Fig. 2 shows what would happen if the concentration transferred was equal to initial mucosal and serosal concentrations. Up to an initial concentration of 60 mM the concentration of acetate leaving the mucosal fluid is greater than the concentration entering the serosal fluid, and both are greater than the initial mucosal and serosal concentration.

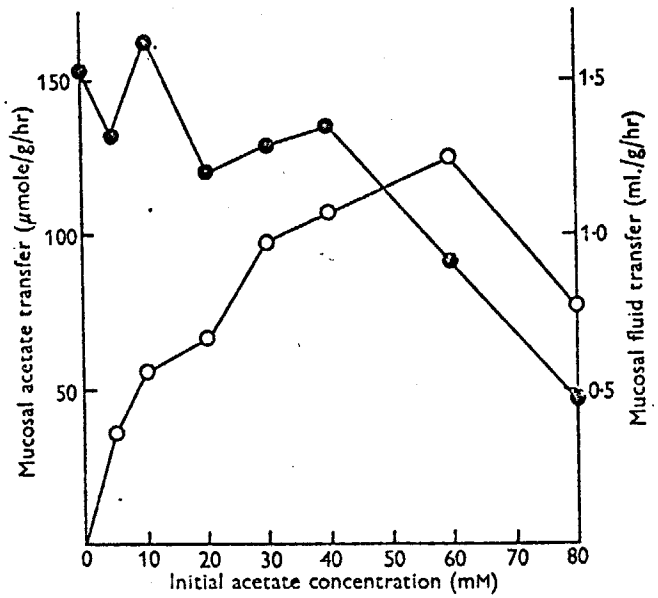

Fig. 1. Effect of initial acetate concentration on mucosal fluid transfer (●) and mucosal acetate transfer (○).

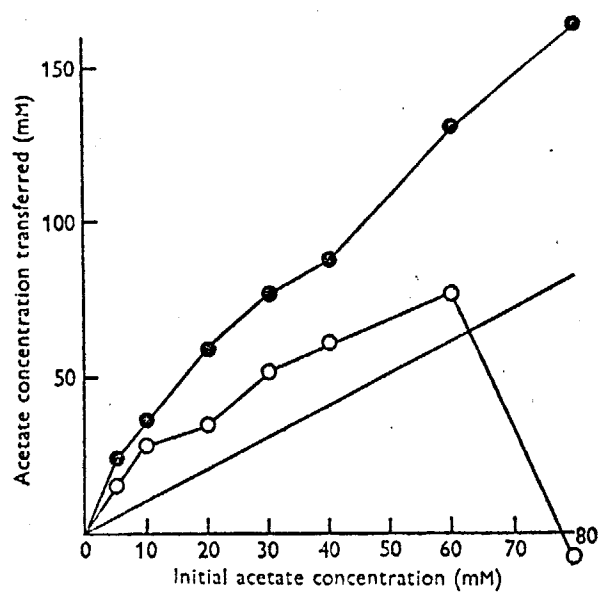

Fig. 2. Effect of initial acetate concentration on the mucosal acetate concentration transferred (●) and serosal acetate concentration transferred (○). The straight line through the origin indicates what would happen if the concentration transferred was equal to the initial concentration.

Thus, up to 60 mM there is active movement of acetate in the sense that concentration of acetate is taking place. At 80 mM there is a dramatic change in the serosal concentration transferred, and this becomes negative. At this concentration acetate moves into the tissue from the serosal fluid, although some fluid is transferred in the reverse direction, i.e. from the intestinal wall into the serosal fluid. The mucosal concentration transferred, however, continues to increase in spite of evidence of inhibition of transfer at high concentrations. Thus a high mucosal concentration transferred is not proof of active concentration of acetate by the intestine. The reason for this is fairly obvious. Initially there is no acetate in the intestinal wall and presumably acetate will diffuse in from both sides. If this

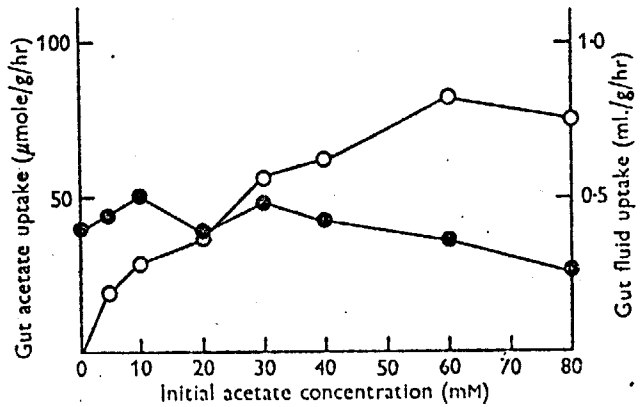

Fig. 3. Effect of initial acetate concentration on gut fluid uptake (●) and gut acetate uptake (○).

is accompanied by only very small fluid movement, as happens with 80 mM acetate, the mucosal concentration transferred may be very high. In contrast, if the serosal concentration transferred is higher than the initial mucosal and serosal concentrations, this is good evidence of concentration of a substance by the intestine.

Figure 3 illustrates the acetate and fluid uptake by the gut and shows that up to an initial concentration of 60 mM the fluid uptake by the intestine remains fairly constant, while the gut acetate uptake is roughly proportional to the initial concentration. Comparison of Fig. 1 with Fig. 3 shows that at these concentrations the gut wall retains about one third of the fluid transferred from the mucosal side, and slightly more than one half of the acetate. The figures are given in Table 6. The proportion of water and acetate retained in the tissues remains remarkably constant in each case until a high initial concentration of acetate (80 mM) is used, when the proportion retained rises sharply. At this stage the amount of both acetate and fluid passing into the sac is diminished much more than

the amount retained by the intestine, and this is shown in Table 6 by an increase in the value of the fraction of mucosal transfer retained in the tissue.

TABLE 6. The fraction of mucosal transfer of acetate and water retained in the gut wall at different initial acetate concn. The figures are average values for a number of experiments. Duration of experiments 60 min

Initial acetate concn. (mM)	Number of experiments	Fraction of mucosal transfer retained in gut wall	
		Fluid	Acetate
5·2	5	0·33	0·59
10·1	3	0·31	0·50
20·2	13	0·32	0·55
30·1	5	0·37	0·57
40·8	10	0·31	0·52
57·5	6	0·36	0·65
80·0	3	0·66	1·00

Michaelis–Menten kinetics

If a substance is transferred by an active process one possibility is that at least one stage in the process may be the combination of the substance with a carrier or an enzyme, and if this stage is rate-limiting then the kinetics of the whole process might conform to the type described by Michaelis & Menten (1913). In testing this the initial concentration and the rate of mucosal transfer have been used. Since there is obvious evidence of inhibition at 80 mM, values at this concentration were omitted from consideration. One of the standard methods of investigating Michaelis–Menten kinetics is that used by Lineweaver & Burk (1934) in which the reciprocals of the concentration and rate are plotted. A weighted regression analysis of the reciprocals of concentration and rate gave a regression equation:

$$y = 0{\cdot}199x + 0{\cdot}0044,$$

where y is the reciprocal of the rate (expressed as μmole/g wet wt. tissue/hr) and x of the concentration (expressed as mM). The intercept on the abscissa was $-0{\cdot}022$, and the negative reciprocal of this (Michaelis constant) is 45 mM. The 95% confidence limits of this value were 37–57 mM. Tests for curvilinearity did not suggest that a straight line was not the best fit for the experimental points.

Transfer of other fatty acids

Experiments were carried out with different concentrations of the other fatty acids, and the mucosal and serosal transfers *in vitro* are shown in Fig. 4. This shows (1) that inhibitory effects are produced by all the fatty acids tested, (2) that the concentration necessary to cause inhibition

decreases with increasing length of chain, (3) that there is no obvious difference in the rate of transfer of odd and even members of the series, and (4), that the inhibitory effects are much more marked on serosal transfer than on mucosal transfer. When mucosal transfer is reduced, a larger fraction of the total serosal transfer is retained in the gut wall, so that what spills over into the sac contents (serosal transfer) is reduced much more, relatively, than the mucosal transfer.

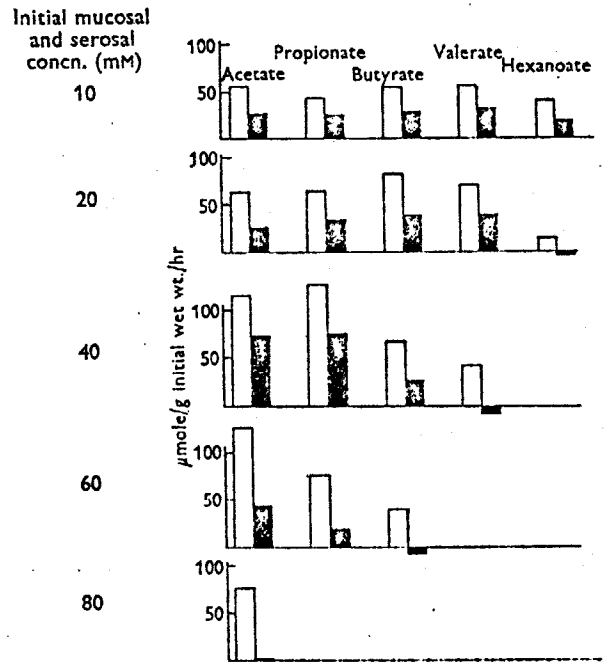

Fig. 4. Relation between length of carbon chain and transfer of fatty acid at various concentrations. The mucosal transfer is shown by white rectangles and the serosal transfer by black rectangles (μmole/g initial wet wt./hr). The lengths of carbon chain were 2 (acetate), 3 (propionate), 4 (butyrate), 5 (*n*-valerate) and 6 (*n*-hexanoate). Each vertical row of mucosal and serosal transfers gives the values for the fatty acid indicated above the top line.

The inhibitory effect of high concentrations of fatty acids is seen also on the final concentration gradient; and this is shown in Tables 7 and 8. Table 7 gives detailed figures for acetate, including the final mucosal and final serosal concentrations. At low concentrations, i.e. up to 40 mM, the increase in serosal concentration is greater than the decrease in mucosal, owing presumably to the much greater volume of fluid on the mucosal side. At 60 mM the increase in serosal is about equal to the decrease in mucosal, suggesting that a greater proportion of the mucosal transfer is being accumulated in the gut wall, but there is still a positive concentration gradient. At 80 mM the inhibition is obvious, with acetate entering the

gut wall from both mucosal and serosal fluids. The final serosal concentration is less than the initial serosal concentration, and the final concentration gradient is negative. Table 8 gives in summary the results with all the fatty acids, the figures for acetate being included for comparison with the others. Evidence of inhibition at high concentrations is seen in the decrease and eventual reversal of final concentration gradient, and the concentration necessary to cause inhibition decreases with increasing length of carbon chain. This negative concentration gradient has been

TABLE 7. Final concn. of acetate at different initial concn. The figures are average values from a number of experiments. The last column gives the final concn. gradient, which is regarded as positive when the final serosal concn. is higher than the final mucosal concn. Duration of experiments 60 min

No. of experiments	Concentration (mM) Initial mucosal and serosal	Final mucosal	Final serosal	Final concn. gradient (mM)
5	5·2	4·0	8·9	4·9
3	10·1	8·2	17·6	9·4
13	20·2	18·3	25·9	7·6
10	40·8	38·0	48·4	10·4
6	57·5	54·5	61·3	6·8
3	80·0	75·5	70·3	− 5·2

TABLE 8. Effect of initial concn. of fatty acids on the final concn. gradient. The final concn. gradient is the difference between final serosal and final mucosal concn. It is considered positive when the final serosal is greater than the final mucosal. Duration of experiments 60 min

Initial concn. (mM)	Final concentration gradient (mM) Acetate	Propionate	Butyrate	Valerate	Hexanoate
5	4·9	6·0	4·6	5·9	5·9
10	9·4	7·8	8·6	12·5	6·0
20	7·6	12·2	12·3	15·2	−4·7
40	10·4	12·0	5·4	− 8·2	—
60	6·8	0·9	− 8·7	—	—
80	− 5·2	—	—	—	—

obtained by Smyth & Taylor (1958) in studying the effect of 2:4-dinitrophenol on fatty acid transfer, by Parsons *et al.* (1958) in studying the effect of phlorrhizin on glucose transfer, and by Jervis & Smyth (1960) in studying the inhibition of amino-acid transfer. In the absence of an active entry from the mucosal side substrate will probably diffuse into the gut more or less equally from both sides. If complete equilibrium is not achieved during the experimental period the small volume of serosal fluid will result in a greater fall in concentration in the serosal fluid than in the mucosal fluid; and hence a negative final concentration gradient will be obtained.

The inhibitory effects of higher concentrations of fatty acid are observed not only on the transfer of fatty acid, but also on fluid transfer. Details

of this are given in Fig. 5 where it is evident that the concentration necessary to cause inhibition of water transfer also decreased with increasing length of carbon chain.

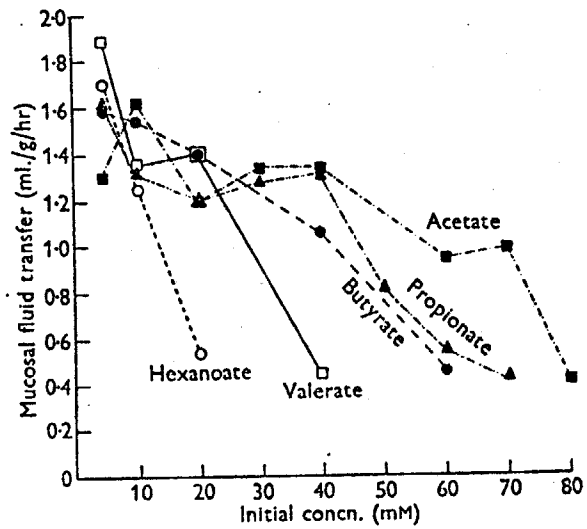

Fig. 5. Effect of initial concentration of a number of fatty acids on the mucosal fluid transfer.

Experiments in vivo

The comparison of rates of absorption of different fatty acids was also studied *in vivo*, by comparing the amounts of fatty acid disappearing from the intestine of the anaesthetized rat in two successive periods in one animal. In each period 50 ml. saline containing fatty acid was circulated through the intestine. In some experiments acetate was present in the intestine during both periods; in other experiments acetate was present during one period, but a different fatty acid was present during the other period. The results are shown in Table 9. This gives the total amount of fatty acid absorbed during the first period and during the second period. The third column of figures shows the ratio of the amount of fatty acid absorbed in the second period to that absorbed in the first period. When acetate was used for both periods the amount absorbed in the second was about 90 % of that in the first. A similar figure for glucose was found by Jervis *et al.* (1956) and for some amino acids by Jervis & Smyth (1959*a*). The last column gives the relative rate of the fatty acid other than acetate, compared with the rate for acetate. It is calculated by assuming that a rate during the second period of 90 % of that of the first period represents equal capacity for absorption for the two fatty acids. The rate for acetate is then considered to be 1, and the figure in the last column is the rate

relative to this of the other fatty acid studied. For example, in the fifth line of the table 365 μmole acetate was absorbed during the first period and 389 μmole propionate during the second period. If acetate had been present in the second period it is assumed that the rate would have been 90 % of 365, i.e. 329 μmole. The ratio 389:329 = 1·18:1, and this figure, given in the last column is therefore a measure of the relative rate of absorption of propionate as compared with acetate. In the seventh line

TABLE 9. Rates of absorption *in vivo* of various fatty acids compared with absorption of acetate. In all cases the initial concn. of fatty acid was 20 mM, and the initial volume 50 ml., the duration of the first and second periods 20 min and the interval between the two periods 20 min. For explanation of the relative rate in the last column see text.

First period		Second period			
Fatty acid	Amount absorbed (μmole) a	Fatty acid	Amount absorbed (μmole) b	$\frac{b}{a} \times 100$	Relative rate of absorption compared with acetate
Acetate	412	Acetate	380	92·2	—
Acetate	452	Acetate	381	84·2	—
Acetate	433	Acetate	396	91·5	—
Acetate	461	Acetate	417	90·5	—
Acetate	365	Propionate	389	100·6	1·18
Acetate	424	Propionate	393	93·0	1·03
Propionate	512	Acetate	434	85·0	1·06
Acetate	537	Butyrate	480	89·2	0·99
Acetate	466	Butyrate	480	102·3	1·14
Butyrate	530	Acetate	414	77·8	1·15
Acetate	432	Valerate	416	96·4	1·07
Acetate	442	Valerate	466	105·5	1·17
Valerate	486	Acetate	382	78·5	1·15
Valerate	455	Acetate	362	79·5	1·13
Acetate	428	Hexanoate	462	107·9	1·20
Hexanoate	557	Acetate	408	73·4	1·23

of the table 512 μmole propionate was absorbed during the first period and 434 μmole acetate during the second. It is assumed that if acetate had been present during the first period the amount absorbed would have been 482 μmole. The ratio 512:482 = 1·06:1. This method can only give approximate comparisons, as it ignores the possible effect of the absorption of one fatty acid on that of a different fatty acid in a subsequent period. None the less, the method offers a relatively simple way of making comparisons and might certainly be expected to show differences between the odd and even members of the series if large differences existed. The results suggest that propionate, butyrate, valerate and hexanoate may all be absorbed at a slightly faster rate than acetate, but there is no evidence of a difference between the odd and even members of the series.

It should be noted that there are important differences between the experiments *in vitro* and *in vivo*. In the former any transfer which occurs

must take place against a concentration gradient, but in the latter this is not the case, and the amount transferred *in vivo* might be expected to be much greater, depending on how much acetate is transferred by diffusion in addition to movement by an active process. Exact comparisons of rates *in vivo* and *in vitro* are not possible, but a rough comparison can be made from the figures in Table 3 and Table 9. In both cases the initial concentration of acetate is 20 mM. In the experiment *in vitro* in Table 3 the mucosal transfer by a sac 30 cm long was 161 μmole/hr. In the experiments *in vivo* in Table 9 the average rate of disappearance of acetate from the intestine was 440 μmole/20 min for the whole jejunum and ileum. The 30 cm sac represents something less than half of the intestine. If the intestine transfers equally well in all parts, the transfer *in vivo* is about four times that *in vitro*. Preliminary experiments at present being carried out suggest that the ileum transfers less well than the jejunum, so that the difference between the rates *in vivo* and *in vitro* might well be greater than this. In the absence of further information it is premature to speculate how much of the difference between the rates *in vivo* and *in vitro* is explained by diffusion, and how much by the different conditions *in vivo* and *in vitro*. One important difference is that the inhibition of fatty acid transfer observed with 20 mM hexanoate *in vitro* is not observed *in vivo*. This aspect of the problem is being further investigated.

DISCUSSION

The experiments described give a more complete picture of the transfers of lower fatty acids than has hitherto been available, and their relation to water transfer. They confirm previous results that the isolated intestine is able to transfer lower fatty acids against a concentration gradient, and they extend the previous observations to include also valeric and hexanoic acids. Use of a more extensive range of fatty acids makes it possible to compare the transfer of odd and even chain fatty acids. Deuel, Hallman & Reifman (1941) suggested a difference in the rate of intestinal absorption of odd and even members of the series, but this has not been confirmed in the present experiments. Instead it has been found that there is a difference *in vitro* depending on the absolute length of the carbon chain. With increase in concentration of fatty acid an inhibitory effect is produced on transfer of both fluid and fatty acid, and the concentration necessary to produce this effect becomes smaller with increasing length of carbon chain. It should be pointed out, however, that the experimental conditions are very different from those used by Deuel *et al.* (1941). These authors fed the fatty acids by mouth to unanaesthetized rats, and the gastric emptying time could play an important part in the results. While the intestinal concentrations were not known, the total amounts of fatty

acid given were about twice the amount used in our experiments *in vivo*. Comparison of the rates of absorption shows that these were of the same order, e.g. Deuel *et al.* (1941) found an average rate of acetate absorption of 62 mg/hr while our figure *in vivo* was 78 mg/hr. The absorptive activity of the intestine is therefore comparable in the two sets of experiments. A possible explanation of the different results is the duration of the experiments. Those of Deuel *et al.* (1941) lasted 1 hr, and it is possible that different rates of removal of odd and even fatty acids from the blood stream by metabolism might cause different rates of absorption. Our experiments lasted only 20 min, and differences in blood concentration might therefore be much smaller. As regards the actual absorption mechanism, differences in surface tension, fat solubility or dissociation of these particular fatty acids are not related to oddness or evenness of the chain, and these are the properties which might conceivably influence absorption rate.

The mechanism of transfer of fatty acids still remains obscure. Smyth & Taylor (1958) suggested the possibility of a common factor in transfer of fatty acid and water, the possible common factor being transfer of sodium. They pointed out however that the effect of DNP and phlorrhizin showed that inhibition of water and fatty acid transfer did not run parallel. In the present experiments the transfer of water and fatty acid also did not necessarily run parallel and could vary independently within certain limits. On the other hand high concentrations of fatty acid inhibited both fatty acid and water transfer, so that there may be some interrelation of the movement of water and fatty acid. One possibility is passive transfer of fatty acid in an active water stream in addition to active transfer of acetate. A similar possibility has been suggested by Smyth & Taylor (1957) for glucose.

The experiments with different concentrations of acetate suggest the existence of a mechanism which can be saturated. It is of some interest to compare the value of the Michaelis constant with those obtained for other substances. Fisher & Parsons (1953*b*) obtained a value of 8–9 mM for glucose absorption *in vitro* and Riklis & Quastel (1958) obtained a value of 7 mM. Jervis & Smyth (1959*b*) obtained values of 10 mM for L-methionine and values of 19–40 mM for D-methionine and D- and L-histidine, and suggested that D-methionine and D- and L-histidine had a low affinity for the transfer mechanism. The value obtained here for acetate would put it also in the class of substances with a low affinity for the transfer mechanism. The interpretation of Michaelis–Menten kinetics in absorption studies requires considerable care. Jervis & Smyth (1959*b*) have pointed out that there may be very large errors in the determination of the Michaelis constant, and apart from this, various authors, e.g. Fisher & Parsons (1953*b*) and Widdas (1952) have stressed that a number of different kinds of

processes may show Michaelis–Menten kinetics. Apart from the question what kinds of processes exhibit Michaelis–Menten kinetics there is the possibility that experimental results obtained with a certain experimental error may suggest kinetic relationships resembling Michaelis–Menten form. The statistical procedures used show that the assumption of a straight-line relationship between the reciprocals of rate and concentration is consistent with the experimental data, but other kinetic relationships approximating only within certain limits to Michaelis–Menten kinetics could permit a similar conclusion. It would be safe to conclude that the results are not inconsistent with the concept of formation of a carrier or participation of an enzyme process, but it would be unwise to go further than this.

Another possibility is that suggested by Brodie & Hogben (1957) that the movement of acetate across the intestinal membrane may depend on differences in pH on the two sides of the membranes. If the un-ionized part of the molecule is in equilibrium on both sides of the membrane, difference in dissociation would lead to different concentrations of fatty acid. According to this view production of pH changes could be an important factor in fatty acid transfer. Since the presence of glucose leads to different pH values on the mucosal and serosal sides (Wilson, 1954) this would provide a possible explanation for the findings of Smyth & Taylor (1958) on the effect of glucose and inhibitors of glucose transfer on fatty acid transfer. It is even possible that the kinetic relationship observed with different concentrations of fatty acids might be related to the effect of these on glucose uptake and lactic acid production. Our experiments do not test this hypothesis, but further experiments are being designed to do so.

The results provide some basis for discussing the interpretation of experiments *in vitro*, and we envisage the following processes taking place. Fluid and solutes are taken up by the epithelial cells from the mucosal fluid, and this probably corresponds to the first process in physiological absorption. The fluid and solutes are then transferred into the subepithelial spaces, and would *in vivo* be removed by the blood or lymph. Removal by the blood stream cannot occur *in vitro* and the fluid therefore accumulates in the tissue spaces in the gut wall. When no more fluid can be accumulated some spills over into the sac contents. In addition, diffusion of solute also occurs from the higher concentration in the gut wall to the lower concentration in the serosal fluid. It seems to us therefore that the process most nearly related to physiological absorption is mucosal transfer either of fluid or of solutes, and that serosal transfer represents only a fraction of this. This fraction is different for different substances, e.g. the figures for Table 4 show that it is 54/92 or 0·59 for propionate and

2·34/3·01 or 0·78 for water. Furthermore, the fraction is not constant even for one substance, as is shown in Table 6. If the activity of the epithelial cells is to be measured by the change in the serosal side, then the introduction of a term 'total serosal transfer' would be necessary, which would include the increase in the sac contents and in the sac wall. It is perhaps useful to relate our terminology to that used by other authors. What has been called in this paper 'serosal transfer' as distinct from 'total serosal transfer', is equivalent to what was termed 'absorption' by Darlington & Quastel (1953) and Wilson (1956*a*); '$Q_{\text{substrate transferred}}$' by Wilson & Wiseman (1954) and Wilson (1956*b*); 'transference' by Wiseman (1955), 'transfer' by Agar, Hird & Sidhu (1953); and 'transport' by Wilson (1956*c*), and Crane & Wilson (1958). According to the concept put forward here these terms all refer only to an unknown fraction of what is transferred through the mucosal cells.

While mucosal transfer is usually preferable to serosal transfer as an index of absorption capacity, there are some important aspects of serosal measurements, and these apply particularly to concentration changes. In cases of small fluid transfer a high mucosal concentration transferred does not necessarily indicate active concentrating capacity, whereas a high serosal concentration transferred does so. The advantage of serosal concentration transferred over final serosal concentration is that it is a more sensitive index, since the final serosal concentration is an average value dependent on the serosal concentration transferred and the initial serosal concentration. Wilson & Wiseman (1954) first used the everted sac to study movement against a concentration gradient. The concept of mucosal transfer and its relation to physiological absorption extend the usefulness of the everted sac, by making it possible to measure how much of a substance can be moved against a particular concentration gradient.

SUMMARY

1. Experiments have been carried out both *in vivo* and *in vitro* on the transfer by the rat small intestine of acetate, propionate, butyrate, valerate and hexanoate.

2. All these fatty acids can be transferred *in vitro* against a concentration gradient.

3. There is no difference between the odd and even members of the series as regards rate of transfer, either *in vivo* or *in vitro*.

4. Higher concentrations of all the fatty acids have an inhibitory effect *in vitro* on transfer of fatty acid and fluid and the concentration necessary to cause this inhibition decreases with increasing length of chain.

5. The significance of mucosal and serosal transfers *in vitro* is discussed and their relation to physiological absorption.

We are indebted to Mr K. Curtis for technical help, and to Mr P. H. Blundell for much valuable assistance with the electronic computation of the results. The expenses of this work were defrayed by grants from the Medical Research Council, and from John Wyeth and Brother Ltd.

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MODULATION THRESHOLDS FOR SINUSOIDAL LIGHT DISTRIBUTIONS ON THE RETINA

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In an optical system, the 'contrast transfer function' describes the relationship between the contrast of test objects whose intensity varies sinusoidally with distance and the contrast of their corresponding images at various spatial frequencies (lines/unit distance). It conveys more information about the characteristics of the system than does the specification of only the resolving power. This approach, widely applied to electrical systems, is strengthened by the formal identity of the transformation equations in diffraction theory between objects and images with those of Fourier analysis and synthesis (Hopkins, 1953). The method can also be applied to the response of a receiving layer, such as photographic film. When a sequence of optical and receiving systems is used in series, the total response of the whole system is deducible from those of its components, provided the principle of linearity holds.

In a physical system when carrying out a sine-wave analysis, one determines the output magnitude for a constant input at various frequencies, but this is not readily feasible in the case of the visual system. Instead, one may reverse the procedure and measure the input required for a constant output, for example, a threshold response. In the present paper data will be presented of the application of such an approach to the retinal and central stages of the visual process, excluding the purely optical ones of the eye.

METHODS

Sinusoidal fringes were created on the retina by a method based on Young's double-slit interference experiment, and this pattern was used to modulate a field of uniform retinal illumination. Light from a compact filament 6 V, 2·75 A lamp (F) is brought to a focus in the plane of a narrow slit, S (Fig. 1*a*). After being collimated by lens L_1 the beam from the slit passes through IF, an interference filter ($\lambda_{max.} = 555$ mμ), and RR, a 2×2 in. (5×5 cm) Ronchi ruling (150 lines/in.; 60 lines/cm). The diffraction image is brought to a focus in plane P_1 by lens L_2. Here is placed a mask that blocks out all but the zero-order and the two first-order images of the diffraction pattern. The zero-order is passed through a

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LIPOID SOLUBILITY, PERMEABILITY AND HEMOLYTIC ACTION OF THE SATURATED FATTY ACIDS.*

By MEYER BODANSKY.

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(Received for publication, June 25, 1928.)

Hemolysis by fatty acids is closely related to the ability of these substances to penetrate the red blood cell membrane. Jacobs (1) has studied hemolysis produced by ammonium salts of the fatty acids and found that these owe their hemolytic effect to the permeability of the corpuscle to ammonia and the fatty acids, both entering the cell with relative ease even in the undissociated form. The accumulation of ammonium salts of the fatty acids inside the corpuscle leads to the osmotic effects which ultimately result in the disintegration of the cell.

The penetration of fatty acids into living tissues has been the subject of many excellent investigations (Loeb, Harvey, Crozier, Taylor, and others), but with the exception of the work of Jacobs with the ammonium salts and the essentially qualitative study of Fühner and Neubauer (2), the permeability of the erythrocyte to fatty acids and the hemolytic action of these substances have received little attention.

Loeb (3) studied the effect of different acids in the formation of artificial fertilization membranes in sea urchin eggs and found the order of effectiveness to be as follows: formic < acetic < propionic < butyric < caprylic < nonylic.

Harvey (4) investigated the penetration rates of different acids into the testis of the "prickly fish," which contains a purple water-soluble pigment sensitive to acids, and found that valeric acid penetrated much more rapidly than any of its lower homologues. Crozier (5) measured the penetration rates of various acids into

* A brief report of these observations was presented at the Ann Arbor meeting of the American Society of Biological Chemists, April, 1928.

the mantle fold of the mollusc, *Chromodoris zebra* and noted the following order of effectiveness: valeric > butyric > propionic > acetic. The effectiveness of formic acid was intermediate between valeric and butyric acids, but in view of the fact that Crozier compared the action of solutions that were 0.01 N in strength, this result is not surprising since the dissociation of formic acid is considerably greater than of its homologues. Crozier has also studied sensory activation by acids (6), using for this work the common earthworm, *Allobophora*. The effectiveness of the various fatty acids was in the order: acetic <propionic < butyric < valeric <formic < caproic < caprylic. A summary of related investigations is to be found in Jacobs' review in Cowdry's "General Cytology" (7).

More recently Taylor (8) determined the threshold concentrations necessary to produce a sour taste and found that for the lower members of the saturated fatty acid series higher concentrations are needed than for the higher members. He found that for solutions of equal pH a high degree of sourness is associated with a high penetration velocity of the undissociated acid or of the anion. It is to be seen therefore that in all the phenomena that have been studied in which primary penetration of the acid is a factor, a given fatty acid is invariably more active than its lower homologues and less active than its higher homologues.

In the present investigation a comparison has been made of the hemolytic action of the members of the saturated series of fatty acids, from formic to capric, including isobutyric, isovaleric, and isocaproic acids. A sufficient number of concentrations in isotonic saline were employed in order to establish for each acid the relationship between hydrogen ion concentration and the rate of hemolysis. In this work, parallel studies were made with dog and human corpuscles. The methods employed have been described in an earlier paper (9).

Even a condensed tabulation of the results that have been accumulated would require a considerable amount of space. The data for the dog corpuscle are therefore represented by means of the curves in Chart I. The data for human corpuscles will be considered separately. These curves bring out for the various acids their relative hemolytic power, and for the individual acids the relation between dilution and the rate of hemolysis. The

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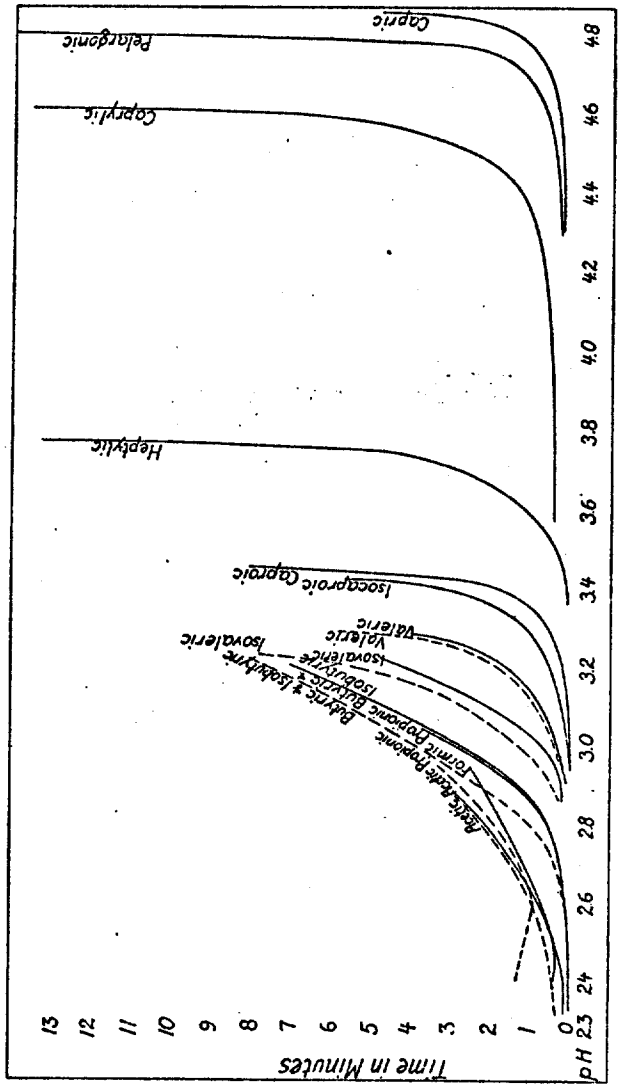

CHART I. Curves showing the relations between pH and the hemolytic action of the fatty acids. The determinations with capric acid were made at 35°. All other determinations were made at 25°.

close parallelism of the results upon which these curves are based and the results of those who have studied the permeability of various tissues to acids makes it difficult to escape the conclusion that the permeability of the fatty acids is intimately related to their hemolytic effect. Formic acid resembles in its action more nearly the inorganic acids than the remaining members of its series. The character of its dilution curve sets it apart from the other fatty acids and the fact that moderate increases in the osmotic pressure of the outside fluid produce relatively little effect on the rate of hemolysis leads one to suspect that primary penetration of the acid is not the main factor involved in the hemolysis by formic acid, but that this acid resembles the inorganic acids in injuring the corpuscles in some other way (9).

Two curves are given for acetic acid. The broken line is based on the results obtained with solutions prepared in isotonic salt solution. The hemolytic action of acetic acid is low. It requires a normal solution to hemolyze a standard cell suspension in about 0.3 minute. Now, a normal solution of acetic acid in water is approximately 4 × isotonic, so that solutions of this acid, more concentrated than 0.25 N, even though prepared in water, are still hypertonic with respect to the cells. In view of the limited range of effectiveness of the acid, the concentrations above 0.25 N could not be excluded from the series, and the fact that these solutions were not isotonic is therefore to be borne in mind in interpreting the solid line for acetic acid, which is based on the results obtained with isotonic solutions of acetic acid of concentrations lower than 0.25 N and with solutions above this concentration that were unavoidably hypertonic. This applies also in the case of propionic acid, but for all the higher acids it was possible to adjust all the necessary concentrations to isotonicity. Two curves are given for propionic acid, the broken line being based on the data for the solutions prepared in saline and the solid line for the isotonic solutions. Two sets of curves are likewise given for butyric, isobutyric, valeric, and isovaleric acids in order to bring out the effect on the rate of hemolysis of small differences in the osmotic pressure of the outside fluid. The remaining curves are based on the results obtained with solutions prepared by dissolving the acid in 0.85 per cent sodium chloride. In the dilutions in which these acids were used, the addition of the acid to the physi-

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side fluid. The remaining curves are
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sodium chloride. In the dilutions in
, the addition of the acid to the physi-

ological salt solution was insufficient to produce any appreciable change in tonicity. A more detailed study of the relation of osmotic pressure to the rate of hemolysis is contemplated.

It will be observed that for a given pH, isovaleric acid is less effective than *n*-valeric acid and isocaproic acid is less effective than *n*-caproic acid in hemolyzing the corpuscles of the dog.

The higher fatty acids are very active hemolytic agents. These acids have a low solubility in water; the statements given in various handbooks that they are insoluble are, however, misleading. For example, a N/1500 solution of pelargonic acid is easily prepared. Pelargonic acid and its lower homologues are liquid at room temperature, and all the measurements with these acids were made at 25°. Capric acid, on the other hand, melts at 31°. The determinations with this acid were therefore made at 35°.

Under approximately uniform physiological conditions the properties of the red blood corpuscle of a given species are apparently such that experimental results of the type considered in this paper reproduce themselves from day to day with a uniformity that is very remarkable. Ponder (10) has observed this sort of consistency in his studies on saponin hemolysis. Corpuscles obtained from different normal individuals have approximately the same average resistance to saponin and the results which Ponder obtained with his own blood from time to time showed little variation. The present author has verified Ponder's assertions using his own blood, the data obtained being practically identical with those given by Ponder. Acids behave similarly in that over a long period of time there is little variation in the resistance of the corpuscles of a given animal towards a given acid. This does not mean, however, that the same mechanism is involved in the hemolysis by these two groups of substances; in fact the mechanisms are probably different.

The constancy in the behavior of the washed corpuscles to a given acid need not lead to the assumption that the permeability of the corpuscle is not subject to variation. The composition of the erythrocyte is far from being constant either physiologically or in a variety of pathological conditions. It is well known, for example, that the lipoids of the corpuscle are altered both qualitatively and quantitatively during fat absorption. Other changes

TABLE I.

*Data Showing Relative Resistance of Dog and Human Corpuscles to Varying Concentrations of Fatty Acids.**

Time for hemolysis in minutes.

Acid	Formic.				Acetic.				Propionic.			Butyric.			Isobutyric.		Valeric.		
Normality†	0.1	0.05	0.02	0.01	1.0	0.5	0.25	0.1	0.5	0.25	0.1	0.5	0.25	0.1	0.5	0.25	0.1	0.05	0.02
pH‡	2.39	2.52	2.73	2.93	2.32	2.47	2.63	2.85	2.58	2.70	2.92	2.49	2.67	2.86	2.52	2.68	2.89	3.06	3.27
Dog	0.36	0.62	1.25	2.30	0.38	0.63	1.17	2.75	0.95	1.25	2.70	<0.05	0.33	1.80	<0.05	0.50	0.07	0.85	5.75
Man	0.26	0.44	1.08	2.33	0.33	0.42	1.00	>4.00	1.22	3.42	>6.00	<0.05	5.75	>12.00	<0.05	7.00	0.11	2.08	>14

Acid	Isovaleric.			Caproic.				Isocaproic.				Heptylic.				Caprylic.		
Normality	0.1	0.05	0.02	0.1	0.05	0.02	0.01	0.1	0.05	0.02	0.01	0.02	0.01	0.005	0.002			
pH	2.86	3.03	3.22	2.92	3.05	3.24	3.42	2.89	3.03	3.22	3.42	3.36	3.43	3.61	3.78	4.33	4.46	4.53
Dog	0.23	1.83	7.75	<0.02	0.05	0.33	3.17	Instantly.	0.05	0.75	5.60	0.09	0.21	1.37	17	1.16	4.00	7.45
Man	0.94	>6.00	>13.0	0.02	0.06	2.00	17.0	0.02	0.07	3.15	>22.0	0.12	0.28	3.50	>35	2.58	12.5	>20

* The solutions used in these determinations were prepared by dissolving the acid in physiological salt solution.

† The normality values are close approximations and represent the concentrations of acid before the addition of the standard cell suspensions.

‡ The pH values represent the actual hydrogen ion concentrations at the beginning of hemolysis and were obtained in separate determinations by diluting the acid solutions with saline in the proportion of 1 cc. of saline to 4 cc. of acid. In determining the time required for complete hemolysis, 0.4 cc. of the standard corpuscle suspension is added to 1.6 cc. of the acid solution.

Action of Fatty Acids

Acid	Isovaleric.			Caproic.								Heptylic.				Caprylic.		
Normality	0.1	0.05	0.02	0.1	0.05	0.02	0.01	0.1	0.05	0.02	0.01	0.02	0.01	0.005	0.002			
pH	2.86	3.03	3.22	2.92	3.05	3.24	3.42	2.89	3.03	3.22	3.42	3.36	3.43	3.61	3.78	4.33	4.46	4.53
Dog	0.23	1.83	7.75	<0.02	0.05	0.33	3.17	Instantly.	0.05	0.75	5.60	0.09	0.21	1.37	17	1.16	4.00	7.45
Man	0.94	>6.00	>13.0	0.02	0.06	2.00	17.0	0.02	0.07	3.15	>22.0	0.12	0.28	3.50	>35	2.58	12.5	>20

* The solutions used in these determinations were prepared by dissolving the acid in physiological salt solution.

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occur in experimental anemia. It is conceivable that changes such as these in the composition of the cell are likely to be associated with variations in the rate of penetration of various substances into the corpuscle.

Variations in permeability of acids would alter somewhat the position and shape of the dilution curves of the type given in Chart I, but despite such variations, the order of effectiveness of the different members of the series as represented by these curves will probably be found to hold under a large variety of conditions for all mammalian corpuscles.

A comparison of hemolysis of human and dog corpuscles by fatty acids has brought out certain distinctions. It will be recalled that human corpuscles are somewhat less resistant to inorganic acids than dog corpuscles. This is also true for formic and acetic acids. The resistance to propionic acid, on the other hand, is somewhat greater in the case of human cells, and for all the higher fatty acids the human corpuscles show a distinctly greater resistance to hemolysis than dog corpuscles. This is brought out by the data outlined in Table I.

The greater resistance of the human corpuscles appears to be related to their higher content of buffer substances. In measuring the shift in potential of the acid solutions during hemolysis, the washed human corpuscles invariably neutralized a greater amount of acid than dog corpuscles. In hemolysis with the lower fatty acids this effect is bound to be of relatively little importance because of the high concentration of acid needed to bring about hemolysis and of the relatively small proportion of this acid neutralized by the constituents of the cells. Yet, even in a 0.1 N solution of acetic acid the human corpuscles exhibit greater resistance than dog corpuscles. In the case of the higher fatty acids where lower concentrations suffice to bring about hemolysis, the difference in the neutralizing capacity of the constituents of the two types of cells may be sufficient to reduce appreciably the amount of available acid and lead to a reduction in the rate of hemolysis. It is to be seen from the data in Table I, as well as from the shape of the curves in Chart I, that a small decrease in the concentration of acid may result in a very pronounced retardation of hemolysis. For example, in the case of caproic acid, at pH 3.4, hemolysis of the standard cell suspension

occurred in about 2 minutes, whereas when the initial pH was 3.45, complete hemolysis did not occur until after an interval of 8 minutes.

The data in Table II have been selected at random from the results obtained in the measurement of hemolysis by means of the potentiometer. The initial pH values of the acid solutions are given, as well as the values at the end of the reaction between the acid and cell constituents. While the differences in the pH shift between the human and dog corpuscles for the various acids is not great, nevertheless differences of like magnitude have a

TABLE II.

*Shift in pH Due to Neutralization of Various Acids by Constituents of Dog and Human Corpuscles.**

Acid	Acetic.	Propionic.	Butyric.	Valeric.	Isovaleric.	Caprylic.
Approximate normality	0.5	0.5	0.1	0.1	0.02	0.001
Dog.						
Initial pH	2.40	2.50	2.77	2.80	3.12	4.30
Final pH	2.58	2.73	3.12	3.11	3.62	5.02
Man.						
Initial pH	2.40	2.50	2.77	2.80	3.12	4.28
Final pH	2.63	2.80	3.18	3.17	3.73	5.20

* To 16 cc. of acid, 4 cc. of the standard red blood cell suspension were added.

considerable effect on the rate of hemolysis, especially for the higher fatty acids, as may be seen by projecting these values on certain portions of the curves given in Chart I.

Mechanism of Penetration of Fatty Acids into Red Blood Corpuscle.

In his study of cell permeability, Harvey (4) did not obtain clear cut evidence either to support or contradict Overton's well known lipoid solubility theory. Harvey states, however, that lipoid solubility, or capillary activity, for the two run more or less parallel, constitutes one of the factors upon which the penetration of acids depends.

Taylor's work on taste (8) has led him to conclude that sourness is not purely a function of the stoichiometric acid concentration, nor of the hydrogen ion concentration. In attempting to find an

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TABLE II.

on of Various Acids by Constituents of Dog
*:man Corpuscles.**

Propionic.	Butyric.	Valeric.	Isovaleric.	Caprylic.
0.5	0.1	0.1	0.02	0.001
2.5	2.77	2.80	3.12	4.30
2.75	3.12	3.11	3.62	5.02
2.50	2.77	2.80	3.12	4.28
2.80	3.18	3.17	3.73	5.20

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Fatty Acids into Red Blood Corpuscle.

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explanation for his results he has assumed that the production of a given degree of sourness is due to the establishment within the cells of the taste bud of a definite hydrogen ion concentration and from his experimental results he has calculated the concentration gradients of equally sour acids. He recalculated similarly the taste data of Paul and Bohnen (see Paul (11)), and from the data of Crozier (5, 6) computed the concentration gradients of acids penetrating *Chromodoris* tissue, as well as the

TABLE III.

Concentration Gradients of Acids Penetrating the Red Blood Corpuscle and Producing Hemolysis of a Standard Cell Suspension in 1 Minute.

Acid.	pH	Total acid concentration.	H^+ ion concentration.	Concentration of undissociated acid outside. (a)	Relative concentration of undissociated acid inside. (b)	Ratio (a):(b).	Ratio (a):(b) when acetic acid = 1.
		$\times 10^{-4}M$	$\times 10^{-6}M$	$\times 10^{-6}M$			
Formic	2.67	23.5	2.14	21.4	1.00	21.4	
Acetic	2.64	286	2.3	284	11.5	24.7	1.00
Propionic	2.86	141	1.4	140	15.2	9.2	0.372
Isobutyric	2.87	122	1.35	121	14.3	8.46	0.342
Butyric	2.87	124	1.35	123	14.5	8.48	0.344
Isovaleric	3.03	51.9	0.93	51	12.6	4.05	0.164
Valeric	3.14	34.1	0.73	33.4	13.4	2.50	0.101
Isocaproic	3.28	19.2	0.53	18.7	14.3	1.31	0.053
Caproic	3.35	14.4	0.45	13.9	14.7	0.95	0.038
Heptylic	3.58	4.9	0.26	4.64	14.7	0.315	0.0128
Caprylic	4.33	0.2	0.047	0.153	14.9	0.0103	0.0004
Pelargonic	4.67	0.06	0.021	0.04	19.4	0.0021	0.000084

concentration gradients causing retraction in the earthworm. The resulting values, not only showed agreement with each other, but resembled Freundlich's data (12) on the relative concentrations of undissociated fatty acids (formic, acetic, propionic, and butyric) which are necessary in order that charcoal may adsorb the same quantity of acid in each case. Accordingly, Taylor suggests that the fatty acids are taken into tissues by an adsorption process.

It is probably unnecessary to postulate an adsorption mechanism, for the results of acid penetration into tissues can be more

fully correlated on the basis of solubility data which are outlined in this paper. In order to compare our results on hemolysis with the data that have been obtained by others in a variety of biological phenomena involving the penetration of acids into tissues, the relative concentration gradients of undissociated acids across the red blood cell membrane, causing hemolysis (*a*) in 1 minute, and (*b*) in 5 minutes, have been determined by the methods suggested in Taylor's paper. The results are outlined in Tables III and IV. The relative concentrations of undissociated acid

TABLE IV.

Concentration Gradients of Acids Penetrating the Red Blood Corpuscle and Producing Hemolysis of Standard Cell Suspension in 5 Minutes.

Acid.	pH	Total acid concentration.	H^+ ion concentration.	Concentration of undissociated acid outside. (a)	Relative concentration of undissociated acid inside. (b)	Ratio (a):(b).	Ratio (a):(b) when butyric acid = 1.
		$\times 10^{-3}M$	$\times 10^{-3}M$	$\times 10^{-3}M$			
Butyric	3.12	39.9	0.76	39.1	14.5	2.70	1.00
Isobutyric	3.12	39.3	0.76	38.5	14.3	2.70	1.00
Isovaleric	3.23	21.1	0.59	20.5	12.6	1.63	0.60
Valeric	3.30	16.2	0.50	15.7	13.4	1.17	0.43
Isocaproic	3.42	10.0	0.38	9.6	14.3	0.67	0.25
Caproic	3.44	9.3	0.36	8.9	14.7	0.60	0.22
Heptylic	3.75	2.3	0.175	2.1	14.7	0.143	0.053
Caprylic	4.58	0.073	0.026	0.047	14.9	0.0032	0.0012
Pelargonic	4.78	0.043	0.017	0.026	19.4	0.0013	0.0005

inside the cell are based on the dissociation constants of the fatty acids, as given in the "Landolt-Börnstein Physikalisch-chemische Tabellen." The present writer has been unable to find data for the dissociation constant of capric acid; the results for this acid are therefore omitted from the tables.

The ratios given in the last column of Tables III and IV may be taken to represent the relative gradients or driving forces which are necessary to cause the various fatty acids to penetrate into the corpuscles of the dog to a comparable degree. When allowance is made for the higher concentrations of acid necessary to

s of solubility data which are outlined compare our results on hemolysis with ained by others in a variety of biologi- the penetration of acids into tissues, radients of undissociated acids across e, causing hemolysis (*a*) in 1 minute, been determined by the methods sug- The results are outlined in Tables concentrations of undissociated acid

TABLE IV.

s Penetrating the Red Blood Corpuscle and andard Cell Suspension in 5 Minutes.

Total acid concentration.	Ion concentration.	Concentration of undissociated acid outside. (a)	Relative concentration of undissociated acid inside. (b)	Ratio (a):(b).	Ratio (a):(b) when butyric acid = 1.
$10^{-3}M$	$\times 10^{-3}M$	$\times 10^{-3}M$			
9.9	0.76	39.1	14.5	2.70	1.00
9.3	0.76	38.5	14.3	2.70	1.00
1.1	0.59	20.5	12.6	1.63	0.60
5.2	0.50	15.7	13.4	1.17	0.43
0.0	0.38	9.6	14.3	0.67	0.25
9.3	0.36	8.9	14.7	0.60	0.22
2.3	0.175	2.1	14.7	0.143	0.053
0.073	0.026	0.047	14.9	0.0032	0.0012
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bring about hemolysis than are required to produce a sour taste or to cause sensory activation as in Crozier's experiments (6), the relations indicated by the data in Tables III and IV nevertheless bear a close resemblance to the relations that have been shown to exist in other biological phenomena which depend upon the primary penetration of the acids into tissues. That the primary penetration of the fatty acids into the erythrocyte depends on their solubility in the lipoids of the cell will now be considered.

It is generally known that the lipoid solubility of the members of the saturated series of fatty acids increases according to the size of the molecule. However, adequate data bearing on the distribution of the fatty acids between water and fatty substances seem to be lacking. In his paper on the permeability of cells to acids, Harvey (4) includes certain data obtained from Wasteneys on the partition coefficients between water and olive oil of several of the fatty acids in 0.01 N concentration.[1] In view of the possible relationship between the penetrability of the fatty acids into the corpuscle and lipoid solubility and because of the lack of solubility data, it seemed desirable to determine the partition coefficients of the fatty acids between water and olive oil. (The same results were obtained with aqueous solutions as with solutions of the fatty acids in saline.)

The solubility of the fatty acids was determined at 23°. 100 cc. portions of the various acids of known concentration (N, 0.1 N, 0.01 N, 0.001 N) were transferred to flasks. After the removal of 25 cc. for the preliminary titration with alkali, 5 cc. of olive oil (Squibb) were added to the remainder; the flasks were tightly stoppered and the contents shaken in a mechanical shaking device for $1\frac{1}{2}$ hours. As a rule the distribution was found to reach equilibrium within 30 minutes. After the oil was allowed to separate, a second 25 cc. portion of the solution was titrated. The oil was then transferred to narrow test-tubes and set aside for 24 hours or longer to insure complete separation of the oily layer. Accurately measured portions of the oil were then dissolved in neutral alcohol and titrated with standard alkali. Suitable control analyses were made for the acid content of the olive oil. Excellent agreement was obtained in nearly all of the experiments

[1] Except for Harvey's citation, this work has not been published, according to a personal communication from Dr. H. Wasteneys.

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TABLE IV.

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...tration.	...ion concentration.	Concentration of undissociated acid outside. (a)	Relative concentration of undissociated acid inside. (b)	Ratio (a):(b).	Ratio (a):(b) when butyric acid = 1.
$10^{-2}M$	$.0^{-2}M$	$\times 10^{-2}M$			
.9	0.76	39.1	14.5	2.70	1.00
.3	0.76	38.5	14.3	2.70	1.00
.1	0.59	20.5	12.6	1.63	0.60
.2	0.50	15.7	13.4	1.17	0.43
.0	0.38	9.6	14.3	0.67	0.25
.3	0.36	8.9	14.7	0.60	0.22
.3	0.175	2.1	14.7	0.143	0.053
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The solubility of the fatty acids was determined at 23°. 100 cc. portions of the various acids of known concentration (N, 0.1 N, 0.01 N, 0.001 N) were transferred to flasks. After the removal of 25 cc. for the preliminary titration with alkali, 5 cc. of olive oil (Squibb) were added to the remainder; the flasks were tightly stoppered and the contents shaken in a mechanical shaking device for 1½ hours. As a rule the distribution was found to reach equilibrium within 30 minutes. After the oil was allowed to separate, a second 25 cc. portion of the solution was titrated. The oil was then transferred to narrow test-tubes and set aside for 24 hours or longer to insure complete separation of the oily layer. Accurately measured portions of the oil were then dissolved in neutral alcohol and titrated with standard alkali. Suitable control analyses were made for the acid content of the olive oil. Excellent agreement was obtained in nearly all of the experiments

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TABLE V.

Solubility of Fatty Acids in Olive Oil.

Acid.	Acid lost from 75 cc. water and recovered in 5 cc. oil.	Acid in 1 cc. water at equilibrium. C_1	Acid in 1 cc. oil at equilibrium. C_2	Oil:water distribution ratio. $C_2:C_1$	Water:oil distribution ratio. $C_1:C_2$
1.0 N.					
	cc.	*cc.*	*cc.*		Formic acid = 1.
Formic	0.11	0.9985	0.022	0.022	1.00
Acetic	0.15	0.998	0.03	0.030	0.73
Propionic	1.05	0.986	0.21	0.213	0.104
Butyric	7.5	0.900	1.5	1.67	0.013
0.1 N.					
					Formic acid = 1.
Formic	0.025	0.9997	0.005	0.005	1.00
Acetic	0.15	0.998	0.03	0.03	0.165
Propionic	0.7	0.991	0.14	0.141	0.035
Isobutyric	3.1	0.959	0.62	0.645	0.0077
Butyric	3.1	0.959	0.62	0.645	0.0077
Isovaleric	11.0	0.853	2.2	2.58	0.0019
Valeric	17.2	0.77	3.45	4.48	0.0011
0.01 N.					
					Propionic acid = 1.
Propionic	0.75	0.99	0.15	0.1515	1.00
Isobutyric	2.15	0.972	0.43	0.443	0.342
Butyric	2.15	0.972	0.43	0.443	0.342
Isovaleric	8.5	0.887	1.7	1.92	0.079
Valeric	11.5	0.847	2.2	2.60	0.058
Isocaproic	26.0	0.653	5.2	7.96	0.019
Caproic	29.0	0.613	5.8	9.47	0.016
Heptylic	57.5	0.233	11.5	49.3	0.003
0.001 N.					
					Butyric acid = 1.
Propionic	0	1.00	0		
Isobutyric	1.5	0.98	0.3	0.306	1.00
Butyric	1.5	0.98	0.3	0.306	1.00
Isovaleric	7.5	0.90	1.5	1.67	0.184
Valeric	9.0	0.88	1.8	2.05	0.150
Isocaproic	16.5	0.78	3.3	4.23	0.072
Caproic	22.5	0.70	4.5	6.43	0.048
Heptylic	40.0	0.47	8.0	17.0	0.018
Caprylic	51.0	0.32	10.2	31.9	0.0095

TABI

ctty .. .ds in Olive Oil.

	Acid in 1 cc. water at equilibrium. C_1	Acid in 1 cc. oil at equilibrium. C_2	Oil:water distribution ratio. $C_2:C_1$	Water:oil distribution ratio. $C_1:C_2$
1.0 N.				
	cc.	cc.		Formic acid = 1.
	0.9985	0.022	0.022	1.00
	0.998	0.03	0.030	0.73
	0.986	0.21	0.213	0.104
	0.900	1.5	1.67	0.013
0.1 N.				
				Formic acid = 1.
25	0.9997	0.005	0.005	1.00
	0.998	0.03	0.03	0.165
	0.991	0.14	0.141	0.035
	0.959	0.62	0.645	0.0077
	0.959	0.62	0.645	0.0077
	0.853	2.2	2.58	0.0019
	0.77	3.45	4.48	0.0011
0.01 N.				
				Propionic acid = 1.
5	[illegible]	0.15	0.1515	1.00
5	[illegible]2	0.43	0.443	0.342
5	0.972	0.43	0.443	0.342
	0.887	1.7	1.92	0.079
	0.847	2.2	2.60	0.058
	0.653	5.2	7.96	0.019
	0.613	5.8	9.47	0.016
	0.233	11.5	49.3	0.003
0.001 N.				
				Butyric acid = 1.
	1.00	0		
	0.98	0.3	0.306	1.00
	0.98	0.3	0.306	1.00
	0.90	1.5	1.67	0.184
	0.88	1.8	2.05	0.150
	0.78	3.3	4.23	0.072
	0.70	4.5	6.43	0.048
	0.47	8.0	17.0	0.018
	0.32	10.2	31.9	0.0095

between the amount of fatty acid lost from the aqueous layer and the amount of fatty acid recovered in the oil. The results, which are based on several series of determinations, are outlined in Table V.

Owing to the low solubility of pelargonic acid, concentrations lower than 0.001 N were employed. With such dilutions, the procedure of titrating the acid solutions before and after shaking with oil was subject to considerable error. The more accurate

TABLE VI.

Solubility of Fatty Acids in Olive Oil.

Acid.	pH shift.		Total acid concentration.		Acid lost from 75 cc. water.	Acid in 1 cc. water at equilibrium. C_1	Acid in 1 cc. oil at equilibrium. C_2	Oil:water distribution ratio. $C_2:C_1$	Water:oil distribution ratio. $C_1:C_2$
	Initial pH.	pH at equilibrium.	Initial $\times 10^{-4}$ M.	At equilibrium $\times 10^{-4}$ M.					
					Cc. 0.000407 N.				
Isocaproic.....	4.15	4.23	0.407	0.291	21.5	0.713	4.30	6.04	0.166
					Cc. 0.000375 N.				
Heptylic.....	4.17	4.35	0.375	0.184	38.2	0.49	7.64	15.6	0.064
					Cc. 0.000443 N.				
Caprylic.....	4.14	4.64	0.443	0.060	64.85	0.135	12.97	96.1	0.014
					Cc. 0.000363 N.				
Pelargonic... ...	4.24	5.02	0.363	0.00934	73.125	0.025	14.625	585	0.0017

procedure was therefore adopted of measuring the change in hydrogen ion concentration. Determinations were made with solutions of isocaproic, heptylic, caprylic, and pelargonic acids of approximately the same concentration. The results are given in Table VI.

An examination of the distribution ratios given in Tables V and VI and comparison of these data with the relative concentration gradients given in Tables III and IV, as well as with the other

data contained in this paper, lead to the conviction that lipoid solubility is an important factor in determining the rate of penetration of the fatty acids into the erythrocyte and the order of effectiveness of the various members of the series in producing hemolysis.

Since the data outlined in this paper are not difficult to correlate, a detailed discussion seems unnecessary, but for the purpose of illustrating how well the results agree, the following example will be considered. It is to be noted that the distribution ratios for butyric and isobutyric acid are identical for the concentrations included in Table V. The hemolysis curves (Chart I) are likewise identical, and accordingly the relative concentration gradients for these acids are the same (Table III and IV). On the other hand, the position occupied by isovaleric acid in the order of effectiveness as a hemolytic agent is intermediate between butyric and *n*-valeric, and isocaproic acid occupies a position intermediate between valeric and *n*-caproic acids. These are precisely the positions occupied by the oil : water (and water : oil) distribution ratios of isovaleric and isocaproic acids.

Incidentally, it is to be observed that the oil : water distribution ratios $\frac{C_2}{C_1}$ for the members of the series above propionic acid increase with increasing concentration of the acids. This indicates that the degree of molecular association of the fatty acids is greater in olive oil than in water. It is likely that this is also true for formic, acetic, and propionic acids, but in view of the low solubility of these in oil, the methods employed in this work were inadequate to bring this out with certainty.

SUMMARY.

The order of effectiveness of the fatty acids in penetrating the red blood corpuscle and producing hemolysis is: acetic < propionic < butyric = isobutyric < isovaleric < valeric < isocaproic < caproic < heptylic < caprylic < pelargonic < capric.

The relations between pH and hemolytic action have been determined for each of these fatty acids and the results represented by means of curves.

Increasing the osmotic concentration of the outside fluid retards

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centration of the outside fluid retards

hemolysis by fatty acids. This shows that osmotic effects are involved in the process.

Formic acid resembles the inorganic acids in its hemolytic action.

Human corpuscles are more resistant to hemolysis by fatty acids than dog corpuscles. This is apparently due to the greater content of buffer substances in washed human corpuscles than in washed dog corpuscles and the neutralization of a greater amount of acid.

Determinations have been made of the relative concentration gradients of the acids, penetrating the corpuscle and producing hemolysis. The relations which are shown to exist in hemolysis resemble closely the relations that have been observed by other workers in a variety of phenomena in plant and animal tissues involving the primary penetration of the acids.

Determinations have been made of the distribution of the fatty acids between water and olive oil. A close parallelism exists between lipoid solubility and the effectiveness of the fatty acids in producing hemolysis.

The writer wishes to express his appreciation to Dr. B. M. Hendrix of this laboratory for a number of valuable suggestions.

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THE DIGESTIBILITY OF ACETIC, PROPIONIC AND BUTYRIC ACIDS BY THE FOWL

35

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SYNOPSIS

Acetic, propionic and butyric acids, fed as the calcium salts at a level so that the diet contained about 2·5 per cent of the fatty acid ion, were completely digested. Examination of the contents of gut sections indicated that each acid was completely removed from the ingesta before this reached Meckel's diverticulum.

INTRODUCTION

Acetic acid is a common constituent of crop contents and propionic and butyric acids have been found in certain crops (Bolton, 1965). It was therefore of interest to determine the fate of each of these acids when they were incorporated in the food.

MATERIALS AND METHODS

Twelve adult fowls of the flock of Brown Leghorns at the Agricultural Research Council's Poultry Research Centre (PRC) were divided at random into three groups of four birds each. They were housed in all-metal layers' cages and a supply of food and water was always available. The diets consisted of PRC Chick Mash (Bolton, 1959) to which one of the acids was added as the calcium salt. The amount added contained about 2·5 per cent of the fatty acid ion. The diets were fed for at least 1 week to obtain equilibrium in the gut. During the second week samples of food, droppings and the contents of five sections of the gut were collected and analysed. Gut-content samples were obtained from birds killed under anaesthesia, care being taken to avoid frightening them before they were anaesthetised so that movements of the gut contents due to muscular spasms were minimised. The crop contents from each cadaver were transferred to a tared beaker. The small intestine was removed and divided at Meckel's deverticulum and each part was divided again at its mid-point. These sections were numbered one to four, starting from the anterior end of the intestine. The contents of the four sections were gently expressed into tared beakers. To obtain sufficient material for analysis the contents of each section were bulked with those of the corresponding section from the other three birds.

Cellulose was estimated by the method of Crampton and Maynard (1938); it is an integral part of the food and is not digested; it was therefore used as datum. The fatty acids were estimated by the chromatographic method of Wiseman and Irvin (1957). The amount of fatty acid per unit of cellulose was calculated for each sample. To facilitate comparisons between the amounts in the contents of each section of the gut, and in the droppings, with those in the food, the values so found were expressed as percentages of those in the food.

RESULTS

The results of three experiments are set out in Table 1. The amount of acetic acid recovered from the crop was nearly 100 per

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TABLE 1

Acetic, propionic and butyric acid contents of ingesta

	Acetic acid	Propionic acid	Butyric acid
Food content (percentage)	2·38	2·37	2·55
Acid found (percentage of that fed)			
Crop	99·0	40·2	59·6
Small intestine			
Section 1	0	2·1	0·5
2	0	1·7	0
3	0	0	0
4	0	0	0
Droppings	1·6	0·4	0

cent of that ingested, but the corresponding percentages for propionic and butyric acids were only 40 and 60 respectively. Acetic acid was not detected in the small intestine but was in the droppings. Propionic acid was detected in the droppings and in small intestine sections 1 and 2, but not in 3 and 4. Butyric acid was detected in small intestine section 1 only.

Examinations post-mortem revealed that when butyrate was present in the diet the crop lining of three out of four birds was seriously eroded; when propionate was present it was slightly eroded in one; the crops of the birds fed acetate were normal. Erosion of crop lining has not been observed during routine examinations of many hundreds of dead birds which had been fed the normal diet at this centre.

DISCUSSION AND CONCLUSIONS

The gut contents contain a rich bacterial flora and for this reason the possibility exists that some of the acids under investigation may have been converted into other compounds. Since the acids were added to the diet as calcium salts, the *p*H of the mash will have been unaffected and the usual changes, *e.g.* hydrolysis of starch to sugar and the proliferation of micro-organisms with the consequent formation of alcohol, lactic and acetic acids, will have taken place. It follows that the percentage of acetic acid in the crop contents may have been increased by these changes. Only trace amounts of propionic and butyric acids have been detected in normal crop contents, hence endogenous production can be regarded as negligible. When a bird is eating, some food passes directly to the proventriculus and the rest goes into the crop where it may remain for some hours. During this time it is possible that water-soluble nutrients are washed out of the crop into the proventriculus, or they may be absorbed by the crop; Soedarmo, Kare and Wasserman (1961) have shown that glucose can be so absorbed. It is not clear whether absorption, leaching or both processes are responsible for the loss of propionic and butyric acids from the crop contents.

The finding of acetic and propionic acids in the droppings, though they were absent from the lower intestine, suggests they may have resulted from bacterial fermentation after voiding; the period of time between collections of droppings was 1 day.

The metabolism of acetate, propionate and butyrate by sheep liver slices has been studied by Leng and Annison (1963). From the distribution of radioactivity in glucose isolated after the incubation of the slices with the substrates they concluded that the 3-carbon propionate could act as a source of glucose, but the incorporation of the 2-carbon acetate and 4-carbon butyrate was consistent with entry into glucose as acetyl-coenzyme A through the tricarboxylic acid cycle. The three acids are normal products of rumen fermentation and the sheep obtains an appreciable proportion of its energy supply from them.

Acetate can be regarded as a normal constituent of the crop contents, but the fowl obtains only a small amount of its energy supply from it; propionic and butyric acids must be regarded as abnormal components.

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Transport of monovalent anions by isolated small intestine of the rat[1]

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CLARKSON, T. W., A. ROTHSTEIN AND A. CROSS. *Transport of monovalent anions by isolated small intestine of the rat.* Am. J. Physiol. 200(4): 781–788. 1961.—Monovalent anions could be divided into three groups, according to relative rates of absorption along the small intestine. *Group 1* (chloride, bromide, iodide, nitrate and thiocyanate) moved at approximately equal rates in both the ileal and the jejunal regions. *Group 2* (bicarbonate, acetate, propionate and phosphate) moved preferentially in the jejunum. *Group 3* (iodate) did not move in either region. Radioisotope flux measurements indicated that the low rate of transport of *group 2* in the ileum was owing to decreased permeability. All anions tested invariably moved down the electrochemical potential gradient. There is a correlation between the three anion groupings and their physicochemical properties suggesting that *group 1* anions move preferentially along aqueous channels, and *group 2* anions diffuse through lipid regions in the undissociated, acidic form. In this respect, both the mucous coating of the mucosal cells and the preferential release of hydrogen ions on the mucosal side of the jejunum, as a result of lactic-acid production, have an essential function in the absorption of *group 2* anions.

SINCE THE PUBLICATION of Hober's work (1), in the 1890's, no comparative study of the absorption of monovalent anions by the small intestine has been reported. Burns and Visscher (2) reported on the effect of various anions on the intestinal absorption of chloride by the dog; more recently, Smyth and Taylor (3), Wilson and Kazyak (4), and Parsons (5) have discussed the movement of fatty acid, chloride and bicarbonate across different preparations of rat small intestine. Shancker, Tocco, Brodie and Hogben (6) presented a comprehensive study of the transport of a series of acidic and basic drugs; they noted that such physical properties as pKa and lipid solubility were important in determining the extent of transport. In general, however, information about anionic absorption has been incidental to the main study, or limited to specific anions.

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This report is on a comparative study of the relative rates of absorption of various anions, made in order to obtain insight into the mechanism of anionic transport by the small intestine.

METHODS

Everted sacs of rat small intestine were prepared as described by Clarkson and Rothstein (7). The volume of mucosal solution was usually 1000 ml, so that no measurable changes in its composition took place during the experiment. In contrast, the initial volume of the serosal solution was 1–2 ml, and changes in its composition and volume provided a sensitive index of intestinal transport. Our experiments were begun with mucosal and serosal solutions of identical composition. Two basic solutions were used: Krebs-Ringer bicarbonate and Krebs-Ringer phosphate, made up according to Umbreit, Burris and Stauffer (8), but with Ca^{++} reduced to 1.2 mM/l. and 10 mM glucose/l. present. Other solutions were simple modifications of these and will be described later. Changes in the concentrations of inulin in the serosal solution were used to assay the net transport of water across the intestine (7). Movements of solutes was calculated from changes in the concentration of solutes and in the volume of the serosal solution.

The following chemical methods were used: sodium and potassium were analyzed with a Beckman DU flame photometer; inulin was analyzed according to the method of Roe, Epstein and Goldstein (9); lactic acid was analyzed by the method of Barker and Summerson (10); and chloride, iodate and nitrate were analyzed polarographically by methods outlined by Koltoff and Lingane (11). The method of Crandall and Anderson (12) was used in measuring thiocyanate. Bicarbonate concentrations were calculated from measurements of total CO_2 by Conway (13) and the pH of the sample, as recorded by a Beckman model G pH meter.

Chloride, bromide and iodide, when present in the same solution, were determined by potentiometric titration with use of silver electrodes. The reagent was 0.01 N silver nitrate in a buffer solution made up of 20 volumes of 0.5 M sodium acetate buffer, pH 5.0, and 80 volumes of anhydrous methanol; 0.2- to 0.3-ml aliquots of the

serosal solution were added, before titration, to 10 ml of the sodium acetate—methanol mixture. Use of this buffer reduced errors owing to coprecipitation, and enabled us to measure mixtures of chloride, bromide, and iodide with a standard error of less than 3%.

Phosphate was measured by the method of Chen, Toribara and Warner (14). The ratio of mono- to dihydrogen phosphate was calculated from the pH of the solution, with use of a value of 6.43 for K_{2A} (15). Samples of solution containing P^{32} were dropped by pipette into pans, allowed to dry and counted under a thin-window Geiger-Müller tube.

Acetate and propionate were measured by the Conway microdiffusion method (13). In this procedure, acetate, liberated as acetic acid, was collected in dilute citrate solution. After back-titration of this solution, to measure the acetate, samples were dropped by pipette into pans, allowed to dry and counted under a thin end-window Geiger-Müller tube. Thus, the specific activity of the C^{14}-labeled acetate could be measured in the same sample, without danger of contamination by other C^{14}-labeled material produced from acetate by intestinal metabolism.

RESULTS

Chloride and Bicarbonate Patterns

When everted segments of rat small intestine are exposed to a Krebs-Ringer bicarbonate solution, water and solutes move from the mucosal to the serosal side of the tissue (7). In our study, this movement is expressed in terms of the calculated composition of the solution entering the serosal compartment of the everted sac. The composition of this solution is determined by dividing each net solute movement by the water movement. The phrase 'the moving solution,' will be used for convenient expression of this movement, but is not intended to imply that a physical transport of bulk solution necessarily occurs.

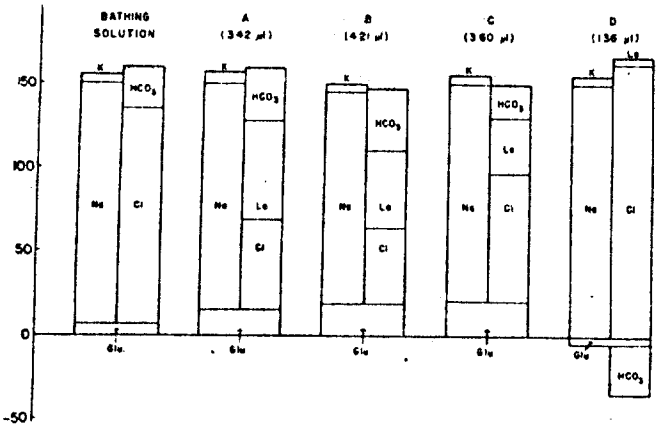

FIG. 1. Intestinal transport in Krebs-Ringer bicarbonate solution containing 10 mM glucose per liter at 37°C. Comparison of the ionic and glucose composition of the moving solution with that of the bathing Krebs-Ringer bicarbonate solution containing 200 mg glucose/100 ml. Values for concentrations in the moving solution were obtained by dividing the net solute transport by the water movement during 60 minutes' incubation at 37°C. A positive value indicates net entry into, and a negative value a net loss from, the serosal solution. The letters, *A*, *B*, *C* and *D*, denote intestinal segments of approximately equal length, consecutively removed from upper part of the jejunum to the ileocecal valve. The net water transport in microliters per milligram dry weight per hour is given in parentheses for each segment of the intestine, so that absolute values may be calculated. Lactate is 'La', glucose, 'Glu'.

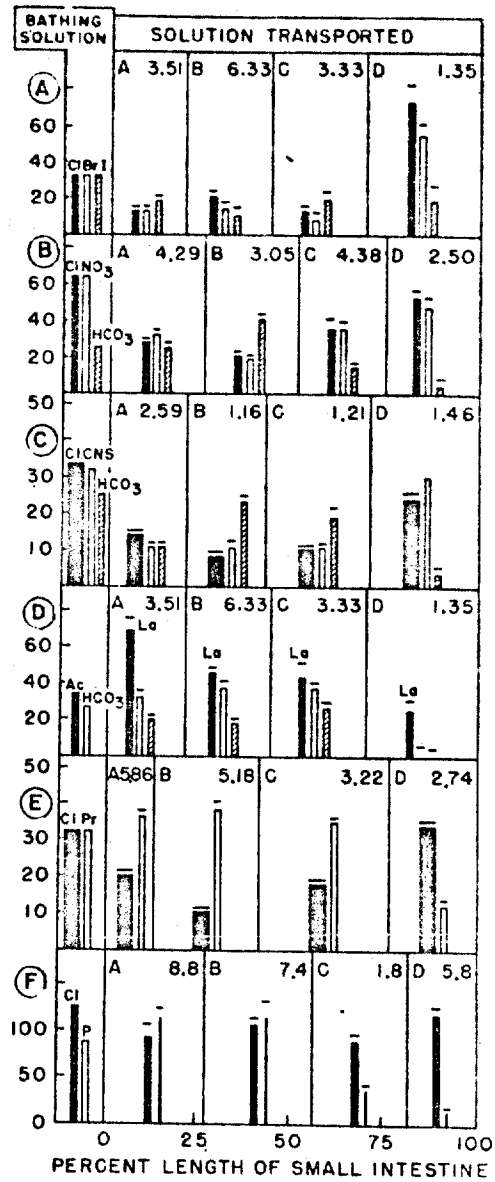

FIG. 2. Comparison of anionic composition of moving and bathing solutions with partial substitution of chloride with another monovalent anion, with data only for pertinent anions quoted. Heights of profiles are proportional to concentrations, but chloride profiles (*C* and *E*) should be multiplied by 3, and phosphate profiles (*F*) divided by 10, to obtain actual concentrations. (Each profile represents the mean of four experiments; vertical distance between top of profile and horizontal bar gives the standard deviation.) Otherwise, the legend for fig. 1 applies. Note: first three anions follow chloride pattern, and second three that of bicarbonate. Acetate, Ac; lactate, La; propionate, Pr; phosphate, P.

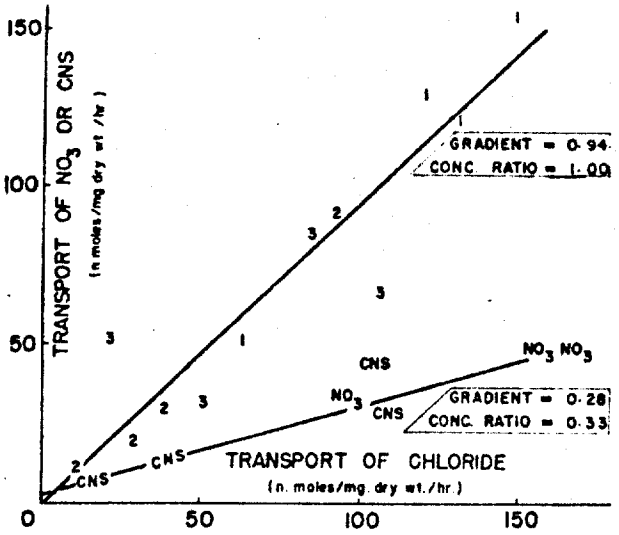

FIG. 3. Relationship between nitrate or thiocyanate transport and chloride transport across both ileal and jejunal segments, when exposed for 60 min. at 37°C to a Krebs-Ringer bicarbonate-glucose solution in which part of the chloride has been replaced by nitrate or thiocyanate. *Upper line* relates nitrate and chloride at 64 mM/l. of bathing solution; *1*, *2*, and *3*, refer to movements in consecutive 1-hr. periods. *Lower line* relates chloride at 100 mM/l. of bathing solution to nitrate or thiocyanate, 30 mM/l., of bathing solution. Note that both gradients correspond closely with concentration ratios. Gradients were calculated by 'least-square' analysis.

The results of a typical experiment, with use of Krebs-Ringer bicarbonate with 200 mg/100 ml glucose as the bathing solution, appear in figure 1. The 'chemical' tonicity of the moving solution (i.e. total concentrations of anion, cation, and glucose) was the same for each segment of the intestine and equaled that of the outside solution.[2] Also, the cationic composition of the moving solution was apparently constant for all four segments. In contrast, the anionic composition of the moving solution varied in the different segments, and did so in a characteristic fashion. Lactate, produced within the mucosal cell (16), appeared as the third anion for *segments A*, *B* and *C*, but little or no lactate was produced by the ileal segment, *D*. The bicarbonate concentration in the moving solution was higher than in the outside solution for *segments A* and *B*. The value in *segment C* was somewhat lower, but, in *segment D*, became negative, indicating a net loss from the serosal solution. Because the total anionic concentration remained constant, the chloride values were reciprocally related to the bicarbonate and lactate concentrations, being minimal in the jejunal segments (*A*, *B* and *C*) and maximal in the ileal segment (*D*). In fact, in *segment D*, chloride accounted for practically all of the anion entering the serosal solution.

[2] Clarkson and Rothstein (7) have noted that measurements of freezing-point depression gave values for the tonicity that were identical with those calculated from the chemical analysis.

Transport of Other Monovalent Anions

Figure 2 gives the results of a series of experiments in which different anions were substituted for a part of the chloride in the bathing solution. The data are presented as profiles in which the concentration of each pertinent anion in the bathing solution is compared with that in the solution moving across each of the four segments of the intestine. Thus, anionic movements in different regions of the intestine may be directly compared, since variations owing to difference in cellular activity and the area of the tissue do not affect these profiles.

Qualitatively, the different anions may be divided into three groups: those which behave like chloride, moving in all parts of the intestine; those which behave like bicarbonate, moving primarily in the jejunal segment; and those which do not move appreciably in any section of the intestine. The results will be discussed in the order of these groupings.

Anions following the chloride pattern. When chloride, bromide and iodide were present in the same concentration (32 mM/l.) in the outside solution (fig. 2*A*), the three anions moved in all four segments of the intestine. The concentrations of bromide and iodide, in general, followed those of chloride in the moving solution. All three anions had the highest concentration in the fluid entering *segment D*, and lowest values in that in the jejunal segments. The iodide profile was, however, significantly lower than that of chloride for *segment D*. This will be discussed later. Nitrate (fig. 2*B*) and thiocyanate (fig. 2*C*) also followed the chloride pattern. The similarity of response suggested that chloride, bromide, iodide, nitrate and thiocyanate might be absorbed by a common mechanism. Quantitative measurements support this suggestion.

The similarity in the movements of nitrate and thiocyanate, as compared with that of chloride, is shown in fig. 3. Nitrate was tested at two concentrations. At a concentration ratio of 1.0:1.0 (nitrate and chloride at 64 mM/l.), the relationship between the ionic movement was linear (upper line, fig. 3), with a gradient of 0.94. At a concentration ratio of 0.33:1.0 (nitrate at 31 mM/l., and chloride at 100 mM/l.), the relationship was again linear (lower line, fig. 3), with a gradient of 0.28. Data

TABLE 1. *Fluxes of Acetate and Chloride Across Small Intestine*

Intestinal Segment	Acetate Fluxes		Chloride Fluxes		Dry Wt., mg
	M/S	S/M	M/S	S/M	
A	243	108	668	239	359
B	241	87	356	178	208
C	117	49	553	250	175
D	52	48	630	350	210

Fluxes are mean values for 60-min. incubation at 37°C in Krebs-Ringer bicarbonate glucose solution containing 100 mM chloride/l. and 30 mM acetate/l. Flux from the mucosal to the serosal side (M/S) and the serosal-to-mucosal flux (S/M) are in n moles/mg dry wt/hr.

from the thiocyanate experiment (fig. 2*C*), in which the concentration ratio was also 3.3:1.0, lie close to the lower line, and were included in the calculation of the gradient by 'least-square' analysis.

The data relating to chloride and nitrate at 64 mM l. were collected hourly for 3 hours, yet deviated little from the linear response (upper line, fig. 3). One may conclude, therefore, that the intestine does not distinguish to any degree among chloride, nitrate, and thiocyanate along its length at high or low rates of absorption, at different concentration ratios (1:1 and 1:3), and in successive periods of absorption (to 3 hr.).

Anions following the bicarbonate pattern. In figure 2 (*D*, *E* and *F*), the anions move in a pattern similar to that of bicarbonate. Acetate concentrations (fig. 2*D*) were maximal in the jejunal segments (*A*, *B* and *C*), and close to zero in the ileum. Lactate, although liberated within the mucosal cell, nevertheless followed the bicarbonate pattern. The data in figure 2*D* are from the same experiments as those in 2*A* so that the acetate and lactate profiles may also be compared directly with that of chloride. Propionate movement (fig. 2*E*) is also contrasted with the movement of chloride; the only difference from bicarbonate and acetate was that the concentration of propionate was higher in the moving solution in *segment D*. A more extensive study would be required to establish the significance of this. Phosphate (fig. 2*F*), as studied in Krebs-Ringer phosphate buffer, also moved reciprocally with respect to chloride.

Characteristic of the bicarbonate group of anions, outlined in the profiles in figure 2, is the fact that their net transport in the ileal segment is close to zero. This absence of net transport could arise either from a general decrease in permeability, in going from the jejunum to the ileum or from an increase in the flux from the serosal to the mucosal side. To distinguish between these alternatives, isotope-labeled buffer was placed on the serosal side, and nonlabeled buffer of identical composition on the mucosal side. As an approximate measure

TABLE 2. *Phosphate Transport by Chemical and P^{32} Measurements*

Intestinal Segment	Phosphorus Transported		Dry Wt.
	Chemical P	P^{32}	
	μM		mg
A-1	6.05	2.58	407
B-1	2.60	1.78	255
C-1	3.00	.66	396
D-1	1.00	.29	666
A-2	5.50	1.20	505
B-2	5.60	2.10	468
C-2	2.80	.20	378
D-2	.10	−.60	250

A positive value denotes net entry into, a negative value a net loss from, the serosal solution. The intestinal segments (*A-1*, etc., from one intestine; *A-2*, etc., from another intestine) were incubated for 60 min. at 37°C in a Krebs-Ringer bicarbonate glucose solution.

TABLE 3. *Fluxes of Inorganic Phosphate Across Small Intestine*

Intestinal Segment	Intestine 1			Intestine 2		
	Mucosal flux	Serosal flux	Tissue P^{32}	Mucosal flux	Serosal flux	Tissue P^{32}
A	32	20	6	105	35	3
B	25	9	6	61	16	6
C	17	5	8	27	7	7
D	6	2	7	10	1	4

The fluxes are mean values for 60-min. incubation at 37°C in a Krebs-Ringer phosphate glucose solution. The flux from the mucosal to the serosal side (M→S) and the serosal-to-mucosal flux (S→M) is expressed in n moles/mg dry wt/hr. The tissue P^{32} (n moles/mg dry wt.) is the amount of P^{32} present in the tissue after 60 min.

of the serosal-to-mucosal flux, the total counts entering the mucosal solution were divided by the mean specific activity on the serosal side. Since we took care to keep the specific activity of the isotope on the mucosal side always at least a factor of 10 below that on the serosal side, we assumed that back-diffusion of the isotope was negligible. Hence, we took the mucosal-to-serosal flux as the sum of the chemically determined net transport and the serosal-to-mucosal flux.

Table 1 shows fluxes for acetate and chloride observed when C^{14}-labeled acetate and Cl^{36} were present in Krebs-Ringer bicarbonate buffer (CH_3COO^- at [illegible] mM l. and Cl^- at 96 mM l.). The mucosal-to-serosal flux for acetate fell from 243 n moles/mg dry wt/hr.[3] in the upper part of the jejunum to 52 n moles/mg dry wt/hr. in the ileum. Similarly, the serosal flux fell from 108 in the jejunum to 48 in the ileum. The chloride fluxes showed no such changes; the ileal fluxes 630 (mucosal→serosal (M→S)) and 350 (serosal→mucosal (S→M)) were similar to the jejunal fluxes, 668 (M→S) and 239 (M→S). Therefore, the slight ileal movements of acetate (fig. 2*D*), must be owing to a general decrease in permeability to this anion, rather than to an increased serosal-to-mucosal flux. The fact that the fluxes of chloride were maintained at about the same level in all regions of the intestine indicates that this permeability change is specific to the bicarbonate group.

Phosphate Movement and Phosphate Fluxes

Further evidence of the specificity of this pattern of flux to the bicarbonate group was obtained from experiments with P^{32}.

The chemical measurements of inorganic phosphate transported into the serosal solution (fig. 2*F*) may include phosphate lost by the tissue. To assess this factor, P^{32}-labeled inorganic phosphate was placed on each side of the tissue, and the phosphate movement was measured both by the chemical method and by the increase in P^{32} counts on the serosal side. Since no P^{32}

[3] n mole = 10^{-9} mole.

TABLE 4. *Titratable Acidity of Serosal Solution**

Intestinal Segment	Serosal pH†	Back Titer as $H_2PO_4^-$	Phosphorus Transported		Dry Wt.
			Chemical P	P^{32}	
			μM		mg
A	6.7	8.8	4.4	4.1	171
B	6.8	8.2	7.6	4.7	220
C	6.9	5.0	1.8	−1.4	248
D	7.0	0.0	−.4	−1.3	186

The values quoted were obtained after 60-min. incubation at 37°C in a Krebs-Ringer phosphate glucose solution. Back-titration of the serosal solution was performed with 0–1 NaOH to the original pH of the solution. These titers were then related to standard curves, based on a similar back-titration of Krebs-Ringer phosphate solution, to which known amounts of $H_2PO_4^-$ has been added. This enabled us to express the back titer of the unknowns in micromoles of $H_2PO_4^-$. * After 1 hr. † The initial serosal pH value was 7.0.

was present in the tissue initially, and since only small amounts accumulate in the tissue during the experiment (table 2), the radioactive determinations should be an accurate measure of the transport process. Results are presented in table. 2 From the difference between the chemical and P^{32} values, as recorded in the second and third vertical columns of table 2, it is obvious that a loss of phosphate from tissue contributes appreciably to the chemical measurements. As shown by the P^{32} values, however, net transport of inorganic phosphate did take place. Typical of the bicarbonate pattern, the maximal transport occurred in the upper and mid-jejunal segments, with essentially none in the ileal segment. In fact, in *segment D* of the second intestine, slight, but significant, negative transport was noted, as is sometimes true for the bicarbonate ion (fig. 1).

To test whether or not the lack of movement across the ileum was owing to low permeability to phosphate, measurements similar to those for the acetate and chloride fluxes, were made, with use of P^{32} label on the serosal side. The results of measurements in two intestines are presented in table 3. In 'intestine 1', the mucosal flux (M/S) falls from 32 n moles/mg dry wt/hr. in the upper jejunum to 6 n moles/mg dry wt/hr. in the lower ileum. Similarly, the serosal flux falls from 20 in the jejunum to 2 in the ileum. Qualitatively similar data were noted for the second intestine. Note that there are no significant differences in the uptake of radiophosphate (*columns 4* and *7*, table 2). Obviously the small or zero net movement of phosphate in the ileum must be owing to a general decrease in permeability rather than to an increased serosal-to-mucosal flux.

In Krebs-Ringer phosphate buffer within the physiological range of pH, inorganic phosphate exists both as $HPO_4^=$ and $H_2PO_4^-$. To what extent is each of these ionic forms transported by the intestine? Phosphate transport across the intestine results in a decrease in the pH of the serosal solution (table 4). These pH changes are greatest in the jejunum, where phosphate transport is also maximal. The total titratable acidity (*column 5*), expressed as μM of $H_2PO_4^-$, however, exceeds that predicted from the transport of inorganic phosphate as $H_2PO_4^-$, as shown from chemical and P^{32} measurements (*columns 3* and *4*, respectively). This excessive acidity probably results from the release of some hydrogen ions from the lactic acid produced in the mucosal cells. Thus, the data in table 3 are compatible with, but by no means proof of, the contention that phosphate moves in the acidic form. The well-established impermeability of the tissue to other divalent ions (e.g. Mg^{++} and $SO_4^=$ (17)) would support the view that $H_2PO_4^-$ is the penetrating species.

Iodate Movement

Iodate was also tested for transport pattern when 30 mM/l. was present in the bathing Krebs-Ringer bicarbonate solution, but no significant movement of this anion across any of the intestinal segments was noted.

Electrochemical Gradients

The small intestine generates an electrical potential during transport of salts and water. From measurements of this potential and from the chemical gradients, one can calculate the total electrochemical gradient acting on the anions in the bathing solutions. The total electrochemical potential was calculated from the equation

$$\frac{\Delta F}{\mathfrak{F}} = \frac{RT}{\mathfrak{F}} \ln \frac{C_m}{C_s} + ZE$$

when $\Delta F/\mathfrak{F}$ is the electrochemical potential gradient between mucosal and serosal solutions, when C_m and C_s are the respective concentrations of the anion in mucosal and serosal solutions; and when E is the electrical potential across the intestine. The significance of $\mathfrak{F}$, R, T, and Z is the same as usual. Also, the ratio of the activity coefficients of the anion in both solutions is assumed to be unity. Values for C_s, recorded in the second vertical column of table 5, are the serosal concentrations after 1 hour of incubation; C_m is identical with the initial value of C_s and remains constant. The E values are also those after 1 hour. Hence, the total electrochemical gradient, expressed as a potential term ($\Delta F/\mathfrak{F}$) in millivolts, is that after 1 hour. The direction of this gradient is most likely unchanged, however, during the entire 60 minutes' incubation, since the electrical potential varied less than 30% from the starting value; the chemical gradient, initially zero, increased to relatively small values, and usually in the direction of the electrical gradient. The notation in table 5 is such that a positive value indicates that the anion is at a higher potential on the mucosal side.

The data in table 5 lend support to a passive mechanism for anionic transfer. The electrochemical potential for all of the anions after 1 hour was always positive and, thus, favorable to net transport from the mucosal to the serosal side. In the last vertical column, note that anionic

TABLE 5. *Electrochemical Gradients Across Small Intestine*

Intestinal Segments	Serosal Conc.	Chemical Potential	Electrical Potential	Electrochem. Potential	Net Anionic Transport
	mM/l.	mv	mv	mv	
Cl^-					
Initial	64				
A	54	4.6	7.2	11.8	121
B	50	6.3	10.3	16.6	63
C	58	2.6	5.8	8.4	150
D	62	0.8	4.2	5.0	132
NO_3^-					
Initial	64				
A	55	4.0	7.2	11.2	139
B	44	10.3	10.3	20.6	50
C	58	2.5	5.8	8.3	154
D	61	1.3	4.2	5.5	120
CNS^-					
Initial	30				
A	24	6.0	7.2	13.2	29
B	26	3.8	7.2	10.2	14
C	26	3.8	7.2	10.2	13
D	30	0.0	5.2	5.2	44
HCO_3^-					
Initial	25				
A	25	0.0	7.2	7.2	106
B	30	−5.1	10.3	5.2	127
C	23	2.5	5.8	8.3	61
D	23	2.5	4.2	7.0	29
CH_3COO^-					
Initial	32				
A	26	5.5	5.1	10.6	135
B	32	0.0	7.0	7.0	154
C	25	6.6	6.2	12.8	68
D	23	8.9	1.0	9.9	2
$CH_3CH_2COO^-$					
Initial	64				
A	33	−0.8	6.6	5.8	209
B	36	−3.2	6.6	3.4	198
C	36	−3.2	5.0	2.8	113
D	28	3.6	2.0	5.6	49
$H_2PO_4^-$					
Initial	5.5				
A	5.7	−1.0	6.2	5.2	26
B	6.2	−3.2	8.0	4.8	34
C	4.6	4.7	4.5	9.2	8
D	4.2	7.2	4.0	11.2	−4
Analytical error	±1.5	±1.0	±0.4	±1.0	±5

Positive values denote that the mucosal solution was at a higher potential than the serosal solution, and a negative value that the mucosal potential was less. These values refer to potential gradients existing across the small intestine at the end of 60-min. incubation at 37°C in a Krebs-Ringer bicarbonate glucose solution with chloride substituted, in part, by another monovalent anion. The phosphate data were obtained with Krebs-Ringer phosphate glucose solution. A positive value for net anion transport (n moles/mg dry wt/hr.) denotes entry into, and a negative value a loss from, the serosal solution.

transfer always occurred in this direction. *Segment D* for $H_2PO_4^-$ (last horizontal line) is an apparent exception. The net chemical transfer—4 μM—is not, however, significantly different from zero.

Closer comparison of the electrochemical potential (*column 5*) and net transport into the serosal solution (*column 6*) supports the conclusion that the ileal segments are relatively impermeable to certain anions. Note that, for Cl^-, NO_3^-, and CNS^- the movement in *segment D* is in the same range as that in *segments A, B* and *C*, in which the electrochemical gradient is the same or even higher. However, with HCO_3^-, CH_3COO^-, $CH_3CH_2COO^-$ and $H_2PO_4^-$, when the electrochemical gradients for *segment D* are at least as large as for jejunal segments, the net transport into the serosal solution is considerably less than in jejunal segments.

It is important to note that it may be misleading to compare absolute figures for net transport (*column 6*) of one anion with those of another. The CNS^- is present in the bathing medium at about the same concentration (30 mM l.) and subjected to the same electrochemical gradient as HCO_3^-, CH_3COO^- and $CH_3CH_2COO^-$, yet these anions have a much greater net transport in *segments A, B* and *C* than does CNS^-. In the CNS^- experiment, however, the transport of other ions was also reduced, so that Cl^- and CNS^- were still transported in proportion to their ratios of concentration in the bathing media (fig. 3).

DISCUSSION

Although it is not our purpose to discuss the energetics of anionic transfer in any detail, a brief review of the literature and of our own data is relevant to the ensuing discussion. The evidence suggests that anionic transfer across the small intestine is essentially by passive diffusion. First, within the Cl^- and HCO_3^- groups, the intestine showed little specificity toward any of the anions tested (figs. 2 and 3). Second, the electrical potential across the intestine was not significantly changed when Cl^- was replaced partly or completely by NO_3^-, or when HCO_3^- was replaced by additional Cl^- (Clarkson and Rothstein, unpublished observations). Third, none of the anions tested moved against its activity gradients. This behavior is in strong contrast with the movement of monovalent cations (7), in which specificity toward sodium was high; transmembrane potential, if sodium were replaced by other cations, fell; and sodium was transported against significant electrochemical gradients.

Our data do not exclude the possibility that a small part of anionic transfer may proceed by a more specialized mechanism. Active transport for iodide has been postulated by Acland and Illman (18) and by Pastan (19), who stated that this anion was actively secreted into the lumen in the mid-jejunal region of the small intestine. Acland reported, however, that passive diffusion predominated over active secretion when external concentrations of iodide greater than 10^{-3} M were used. The concentrations of iodide used in our experiments (30×10^{-3} M) were at least 30 times those used by those authors (10^{-3}–10^{-4} by Acland and Illman, and carrier free by Pastan). Curran and Soloman (20) suggested that an active transport of chloride across the ileum accounted for the difference between observed flux ratios and those predicted on the basis of passive diffusion. In fact, other factors may also contribute to discrepancies

between predicted and observed flux ratios. As suggested by Ussing (21), solvent drag forces arising from net water transfer would tend to increase the value of the observed flux ratio, whereas the presence of an exchange diffusion component would lower the flux ratio (22). Thus, a precise interpretation of the chloride flux ratios across the small intestine is not possible at this time. The fact that the iodide movement was quantitatively lower than chloride (fig. 2A) does suggest, however, the contribution of a specific mechanism, possibly involving a carrier, to the total net transport of chloride. Apart from this possibility, the data in this report are compatible with anionic transport's being dominated by passive diffusion.

Our data and those in the literature suggest that all the monovalent anions studied may be divided into three groups. The entire intestine is permeable to *group 1* (Cl^-, Br^-, I^-, NO_3^- and CNS^-); only the jejunal segments are permeable to *group 2* (HCO_3^-, CH_3COO^-, $CH_3CH_2COO^-$, $CH_3(CH_3)_2COO^-$, $H_2PO_4^-$); and the entire intestine is relatively impermeable to the *group 3* (IO_3^- and $CH_3CH(OH)COO^-$).

The reciprocal relationship between the concentration profiles of Cl^- and HCO_3^- (fig. 2) has been noted in both *in vivo* and *in vitro* properties of the intestine (4, 5). According to our data, Br^-, I^-, NO_3^- and CNS^- behave in a manner similar to that of Cl^-, i.e. they are absorbed throughout the intestine. Acetate, propionate, and phosphate, like bicarbonate, are on the other hand, transported in the jejunal segments, but not in the ileum. Smyth and Taylor (23) placed acetate, propionate, butyrate and phosphate in the same group after noting that the concentrations of these anions were higher in the moving, than in the bathing, solution. In our experiments, concentrations of acetate, propionate, and phosphate in the jejunal segments were similarly higher (fig. 2). The similarity between Smyth and Taylor's data and ours suggests that butyrate may also belong to the bicarbonate group. Since the results of studies of acetate and phosphate fluxes indicate that ileal absorption does not take place owing to low permeability to these anions, the same explanation may also apply to butyrate.

Wilson's data (16) indicate that when lactate is added to the mucosal side, there is little net transport of the anion to the serosal side. For this reason, lactate is classified with iodate in *group 3*, for it does not move significantly in either the jejunal or the ileal segment. This is particularly interesting in view of the release of lactate across the serosal surface of the cells (24). Leaf (25) has reported that permeability to lactate is much greater at the serosal, than at the mucosal surface of the toad's bladder. Clearly, the barrier of permeability must lie at the mucosal surface of the intestine. The release of lactate does, however, follow the same profile as that of bicarbonate, being released by the jejunal, but not by the ileal, segments (fig. 2*d*), probably reflecting the greater metabolic activity of the jejunal, as compared with the ileal, cells.

TABLE 6. *Physicochemical Properties of Monovalent Anions*

Location of Transport	Anion	Electrical Mobility*	pK†	Distribution Ratio Ether/Water K‡
Group 1	Cl^-	76	<1	<.1
	Br^-	78	<1	<.1
All regions	I^-	77	<1	<.1
	NO_3^-	71	<1	.03
	CNS^-	66	<1	<.1
Group 2	HCO_3^-	44	6.4	
	CH_3COO^-	41	4.8	0.5
Jejunal region	$CH_3CH_2COO^-$	36	4.9	1.5
	$CH_3(CH_2)_2COO^-$	<36	4.8	5.1
	$H_2PO_4^-$	40	1.8	
Group 3	IO_3^-	39	0.8	
Not transported	$CH_3CH(OH)COO^-$		3.8	.08

* Expressed as ohm^{-1} cm^2 $equivalent^{-1}$ at 25°C. † As quoted from Harned and Owen (15). ‡ $K^1 = (A)_B/(A)_{H_2O}$ when (A) is the equilibrium concentration of the distributed acid A in phase B (diethyl ether) and $(A)_{H_2O}$ the equilibrium concentration in water. $(A)_B$ = 0.01 M for all values quoted in this column. Data were taken from the International Critical Table (29).

If permeability along determines the patterns of anionic movement across the small intestine, most likely some simple physicochemical properties may distinguish the three groups of anions. In table 6, comparisons are made of electrical mobility, acid dissociation constant, and partition coefficient (ether and water). Note that those anions that travel throughout the small intestine (*group 1*) are small (i.e. have high electrical mobility), are anions of strong acids, and are lipid insoluble. Anions moving preferentially in the jejunum, however, are large (have low electrical mobility) and are anions of weak acids ($H_2PO_4^-$ being a borderline acid), the not dissociated forms of which have appreciable solubility in lipids (as evidenced by their distribution coefficients between ether and water). This class (*group 2*) may compare with the group of acid drugs which, according to Schanker and co-workers, (6) have pKa values greater than 3 and lipid solubility in the nondissociated form. Finally, with reference to the two anions in *group 3*, iodate is too large to fall into *group 1* (electrical mobility, 39), and has a pK value too low for *group 2*; lactate, also presumably too large for *group 1*, has a pK value compatible with that of *group 2*, but its lipid solubility is relatively low.

The observed relation between the movement of the monovalent anions and their physicochemical properties leads to consideration of the structure of the membrane. These movements are compatible with the membrane on the luminal face of the mucosal cells, having a mosaic-like structure, with areas of lipid material interspersed with small aqueous pores. This is, in fact, identical with the lipid-sieve theory put forth by Collander and Borlund (26) on the basis of studies of the rate of penetra-

tion of solutes in various cell types. Thus, for the mucosal cells of the small intestine, *group 1* anions would move predominantly along the aqueous channels, which are not available to the larger (*group 2* and *group 3*) anions. The rate of transport of *group 2* anions would depend upon the capacity of the nondissociated acid form to dissolve in the lipid regions of the membrane. The proportion of nondissociated acid available for diffusion should be determined by the pK value of the acid and the pH value of the solution at the surface of the mucosal cells. On this basis, one can understand the localization of *group 2* anionic transport to the jejunum, for it is likely that the pH value at the surface of the mucosal cells of the jejunum is much lower than that of the ileum. Much more lactic acid is produced in the jejunum than in the ileum, and the hydrogen ion is preferentially released on the mucosal side (16). This fact, along with the unique physicochemical properties of the mucous coating on the luminal surface of the intestine (27), would allow a considerable pH gradient to be established between the surface of the epithelial cells and the bulk solution. Indeed, Hogben *et al.* (28) have calculated, on the basis of absorption of acid drugs by the small intestine, that the layer of solution immediately adjacent to the mucosal cells must have a pH value as low as 5.0.

The mosaic pattern might not reside in the membrane of individual cells. For example, the aqueous channels might be located between the mucosal cells (in the intercellular cement) and the lipid channel through the cellular membrane; or the mosaic might be owing to different aggregates of cells in the mucosa, although no anatomical evidence supports such a conclusion.

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The Effect on Performance and Bacterial Flora of Lactic acid, Propionic acid, Calcium propionate and Calcium acrylate in the Drinking Water of Weaned Pigs

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SUMMARY.—All the substances used were effective in controlling haemolytic *Escherichia coli* and reducing counts of non-haemolytic *E. coli* in the duodenum and jejunum. The effects of treatments were only apparent during the treatment period and disappeared after treatments had ceased. The treatments were administered in the drinking water which was given on top of the food. Consequently the efficiency of the treatments, both bacteriologically and in terms of growth and performance, was dependant on their acceptability. Lactic acid appeared to be most acceptable and was the only treatment to give consistently faster growth and more efficient live-weight gains than controls.

Introduction

SUPPLEMENTATION of the diet of weaned pigs with lactic acid has been reported to improve growth performance and reduce the count of *E. coli* in the gut (Kershaw, Luscombe & Cole, 1966). This paper reports a further study of this compound and of three others administered in the drinking water. The results

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Tumours of the Urinary Bladder and Liver Associated with Enzootic Haematuria in Kenyan Cattle. —Concluded.

haematuria, we have not found any report of changes in the liver. The tumours in the liver were haemangiomas quite similar to those seen in the urinary bladder and it is possible that the agent inducing haemangioma of the urinary bladder also induced the haemangioma of the liver in these cattle. Mugera (1968) in his study of bovine neoplasms in Kenya reported no haemangioma of the liver except those associated with urinary bladder tumours.

The aetiology of enzootic haematuria and urinary bladder tumour in cattle is not known. Numerous factors have been associated with the cause of enzootic haematuria (Heeschen, 1959; Pamukçu, 1955; Pamukçu, Goksoy & Pierce, 1957). In East Africa, as in many other countries, the ingestion of bracken fern is commonly suggested as a possible cause of enzootic haematuria. There is some experimental evidence to support this view. For example, Rosenberger and Heeschen (1960) produced haematuria in five cows by feeding bracken fern for 15 months. Pamukçu *et al.* (1967) induced transitional cell papilloma, a fibroma, capillary haemangiomas, and transitional or squamous cell carcinoma of the urinary bladder in cattle by feeding bracken fern. Georgiev, Urigasov, Antonov and Dimitrov (1963) obtained an extract from the urine of cattle fed hay from haematuria districts, which, when introduced into the urinary bladder of dogs, produced changes similar to a haemangioma. Applications of the same extract to the skin of white mice produced papillomas.

The animals examined in this report came from farms where bracken fern is present and it is possible that these had low doses of bracken fern for a long time and that the lesions in both the urinary bladder and liver were a result of chronic fern poisoning. Mugera, Nderito and Sorheim (1968) in their survey of the tumours of the urinary bladder in Zebu cattle in Kenya have seen a high incidence of urinary bladder tumour in Zebu cattle with no lesions in the liver. These Zebu cattle come from areas where there are no bracken fern. The tumours are mainly papillomas and transition or squamous cells carcinomas and some of the tumours are similar to those described by Brobst and Olson (1965) as being experimentally induced by the bovine cutaneous papilloma agent. Olson, Pamukçu and Brost (1965) induced papillomas of the skin, virgina and urinary bladder with suspension of spontaneous urinary bladder tumours, and isolated a papilloma-like virus from the tumours.

It could therefore be postulated that in Kenya, bracken fern may be associated with the aetiology of urinary bladder tumour and liver tumours in the Kenya highlands, but that a virus or other agent may be involved with the aetiology of the urinary bladder tumour in Zebu cattle in areas where no liver lesions are seen, and where no bracken fern is present.

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obtained previously with lactic acid may have been due either to a specific effect of this substance or to its acidic properties. Accordingly it was decided to include another acid in the present trial and propionic acid was used. Its salt, calcium propionate was also included as a treatment. Sodium acrylate had been reported to influence performance and gut flora of chicks (Sieburth, 1961; White-Stevens, Pennock, Stokstad & Sieburth, 1962) and while pigs showed some response, its effects were not as marked as lactic acid (Kershaw *et al.*, 1966). It was decided, therefore, to test the effects of calcium acrylate as the fourth treatment.

The opportunity also arose to accommodate half the pigs in a new experimental house which had not been occupied before. The other half were put in a house which had been used for the last 12 years and had been occupied on different occasions over the last three years by pigs suffering from post weaning scours. It was intended that the new house would simulate good husbandry conditions and the old house, which had not been disinfected after its last batch of pigs, would simulate poor husbandry conditions.

Materials and Methods

The experiment was conducted in two parts, as a growth trial and as a bacteriological study.

Treatments

For the first four weeks after weaning, at approximately 56 days of age, pigs were subjected to one of four treatments or left as untreated controls. The treatments, which were applied in the drinking water, were as follows:—

Treatment A Water (Control)
Treatment B Water + 0·8 per cent. lactic acid
Treatment C Water + 0·8 per cent. propionic acid
Treatment D Water + calcium propionate (to give 0·8 per cent. propionate)
Treatment E Water + calcium acrylate (to give 0·8 per cent. acrylate)

Pigs were housed communally in groups of four or five, but fed separately in individual feeders. The treatments were applied in the drinking water which was given twice daily on top of the meal. Pigs were given 2·5 lb. liquid per lb. meal and the whole was offered as a mash with no other water available. Pigs were fed an amount of meal according to their live-weight and the composition and scale of feeding of the diets are given in Table I.

Growth Trial

The trial was conducted using 60 pigs arranged as 12 randomised blocks of five pigs each. Pigs were allocated to blocks on the basis of similarity of live-weight, sex and parentage and within blocks randomly allocated to treatments. Blocks were divided equally between the old and the new house and there were equal numbers of male and female blocks. Treatments were applied for the four-week post weaning period and performance was also measured for a further period of eight weeks after treatments had ceased.

Bacteriological study

A pig from each of nine litters was killed for examination before treatments were applied in order to obtain baseline data on the bacteriological status at weaning of the animals used in the bacteriological study and the growth trial. A further four pigs were put on each treatment (half in the new house and half in the old house) and slaughtered for examination at the end of the four-week treatment period. Samples for bacteriological examination were taken from the duodenum and jejunum of pigs killed at weaning and at the end of the treatment period. Rectal swabs were also taken from these pigs and from a further six pigs on each treatment in the growth trial, three weeks after treatments had ceased.

Contents of the duodenum and jejunum were removed aseptically for quantitative estimation and haemolytic reaction of *E. coli*. Samples were stored with ice prior to isolation and counting by the Miles and Misra technique. Rectal swabs were taken as aseptically as possible on sterile swabs. Haemolytic reaction was tested and growth from blood agar plates expressed as a semi-quantitative score.

Results

Growth Trial

The results of the growth trial are given in Table II.

For the four-week period after weaning, when treatments were applied, the only pigs to have faster growth rates than the untreated controls, were those fed lactic acid, but the 9 per cent. difference was not

TABLE IA
THE COMPOSITION AND ANALYSIS OF THE DIETS

	A*	B†
Ingredients per cent.		
Barley	83·0	84·5
Fish meal	7·5	7·5
Soya bean meal	7·5	7·5
Minerals and Vitamins	0·25	0·25
Salt	0·25	0·25
Ground Limestone	1·5	—
Analysis (of air dry feed)		
‡Digestible energy (Kcal per lb.)	1390	1410
Crude protein per cent.	16·3	16·3
Calcium per cent.	0·97	0·55
Phosphorus per cent.	0·68	0·65

TABLE IB
SCALE OF FEEDING OF DIET A

Live-weight (lb.)	lb. per feed per day
40	2
50	2½
60	3
70	3½
80	3¾
90	4

* Diet A fed to Control, lactic acid and propionic acid groups at the levels shown in Table Ib.

† Diet B fed to calcium propionate and calcium acrylate groups at a level to give digestible energy and calcium intake similar to diet A.

‡ Calculated.

TABLE II
THE EFFECT OF TREATMENTS ON GROWTH AND PERFORMANCE DURING THE TREATMENT AND POST-TREATMENT PERIODS OF THE GROWTH TRIAL

Treatment	Control	Lactic acid	Propionic acid	Calcium propionate	Calcium acrylate	Significance of differences	Standard error of difference	† Multiple range test	New house	Old house	Significance of difference
Treatment symbol	A	B	C	D	E						
Treatment period (weeks 1-4)											
Feed intake (lb. per day)	2·22	2·26	2·04	2·05	1·91	*	0·107	ECDAB	2·02	2·16	*
Growth rate (lb. per day)	0·83	0·90	0·78	0·76	0·69	*	0·064	EDCAB	0·74	0·85	**
Feed utilisation (lb. feed per lb. gain)	2·72	2·53	2·68	2·70	2·82	N.S.	0·132		2·79	2·59	*
Post-treatment period (weeks 5-12)											
Feed intake (lb. per day)	3·87	3·90	3·75	3·80	3·82	N.S.	0·083		3·74	3·90	**
Growth rate (lb. per day)	1·45	1·47	1·43	1·46	1·46	N.S.	0·129		1·45	1·46	N.S.
Feed utilisation (lb. feed per lb. gain)	2·68	2·65	2·64	2·61	2·62	N.S.	0·063		2·58	2·69	*
Overall period (weeks 1-12)											
Feed intake (lb. per day)	3·33	3·37	3·18	3·21	3·18	N.S.	0·089		3·18	3·32	*
Growth rate (lb. per day)	1·24	1·28	1·21	1·23	1·20	N.S.	0·038		1·21	1·25	*
Feed utilisation (lb. feed per lb. gain)	2·66	2·65	2·63	2·62	2·64	N.S.	0·480		2·61	2·66	N.S.

† Multiple range test (Duncan, 1955) was carried out where a significant "F" value obtained. The difference between any pair of treatment means not underlined by the same line is significant at 5 per cent. level.

significant. There were significant differences among the treatment groups ($P<0·05$) and pigs given calcium acrylate grew significantly less quickly than the controls. Differences in growth rate between treatments were the result of feed intake and feed utilisation differences. Although pigs were offered food twice daily, to a scale based on live-weight, some pigs refused all or part of their allowance. As the treatments were applied in the drinking water which was put on the food, the acceptability of the compounds included in the water affected the amount of food eaten. This occurred particularly on the calcium acrylate treatment during the first week. Feed refusals were also noted in some cases on the propionic acid and calcium propionate treatments. Although there were no significant differences for feed utilisation, during the four-week treatment period, pigs fed lactic acid were noticeably more efficient than the control and treatment groups.

Table II shows that the effects of treatments were observed only during the period in which they were applied. For the eight-week period following the cessation of treatments there were no significant differences for feed intake, growth rate or feed utilisation. Over the whole 12 weeks from weaning to the end of the experiment, significant treatment effects were also absent.

During the treatment period immediately after weaning both control and treated pigs kept in the old house had significantly faster growth rates ($P<0·01$) and also better feed utilisation ($P<0·05$) than pigs in the new house. However, during the eight-week post treatment period there was no significant difference for growth rate but pigs in the new house made significantly more efficient gains.

Bacteriological study

Table III gives bacteriological results, for pigs killed at weaning, of samples taken from the duodenum and jejunum and of rectal swabs. The pigs killed were a sample taken from nine litters used in the growth trial and bacteriological study. The results for littermates of pigs killed later, in the bacteriological study, are also given in Table IV. The results indicated the presence of haemolytic *E. coli* and quantitatively there was a wide range of intestinal counts.

TABLE III
BACTERIOLOGICAL STATUS AT WEANING OF PIGS TAKEN FROM NINE LITTERS USED IN GROWTH TRIAL AND BACTERIOLOGICAL STUDY

Pig No.	Duodenum (mill. *E. coli* per ml. intestinal content)	Jejunum (mill. *E. coli* per ml. intestinal content)	Rectal swab*
271	No growth	34·7	*E. coli, Lactobacillus* sp.
326	0·6	5·5	*E. coli, Bacillus* sp., *Lactobacillus* sp.
324	0·6	14·5	*E. coli, Streptococcus faecalis.*
231	2·2	0·5	*E. coli, Bacillus* sp., *Streptococcus faecalis.*
300	2·8	1·1	*E. coli, Streptococcus faecalis.*
303	2·8	6·0	*E. coli, Lactobacillus* sp., *Staphylococcus albus.*
274	22·1	108·9	*E. coli, Streptococcus faecalis.*
287	39·4	54·8	*E. coli, Lactobacillus* sp.
310	44·2	0·3	*E. coli, Lactobacillus* sp.

N.B. *E. coli* from duodenum, jejunum and rectal swabs were all haemolytic.

* All plates from swabs yielded confluent growth of all organisms isolated.

Results of bacteriological assessment at the end of the four-week treatment period are given in Tables IV and V. Samples from the duodenum and jejunum of control pigs, which had received no treatment, yielded a mixture of haemolytic and non-haemolytic *E. coli* while pigs from all treatment groups yielded only non-haemolytic *E. coli*. Generally, treated pigs had a lower count of organisms in the intestinal contents than

TABLE IV

EFFECT OF TREATMENTS ON *Escherichia coli* POPULATION OF DUODENUM AND JEJUNUM (MILL. ORGANISMS PER ML. INTESTINAL CONTENTS)

	Sow	Pigs killed before treatments applied (*i.e.* at weaning)	Pigs killed at end of Week 4				
			Control	Lactic acid	Propionate acid	Calcium propionate	Calium acrylate
Duodenum							
Old house	229	44·20 (H)	112 (h)	0·30	1·00	5·60	<0·01
	340	0·60 (H)	145 (h)	0·02	0·04	1·70	+
New house	324	0·60 (H)	2·70 (h)	0	2·00	<0·01	3·6
	228	0·08 (H)	0·40 (h)	0	0	0	0
Jejunum							
Old house	229	0·30 (H)	140 (h)	0·20	0·06	6·20	0·08
	340	14·50 (H)	2·30 (h)	0·04	0·10	0·70	+
New house	324	5·50 (H)	0·20 (h)	0	3·40	<0·01	1·40
	228	6·00 (H)	1·50 (h)	0	0·40	0	0

.·. Pig died, having 2,800 mill. organisms per ml. intestinal content in duodenum and 30,600 in jejunum.
H were haemolytic *E. coli* and h a mixture of haemolytic and non-haemolytic strains. For other pigs in which *E. coli* were found there were non-haemolytic strains only.

TABLE V

EFFECT OF TREATMENTS ON *E. coli* AS MEASURED BY RECTAL SWABS AT END OF TREATMENT PERIOD AND THREE WEEKS AFTER TREATMENTS HAD CEASED

	Control	Lactic acid	Propionic acid	Calcium propionate	Calcium acrylate
At end of treatment period (i.e., four weeks post-weaning.)					
Old house	+++	++	+	++	+
	+++	++	++	++	*
New house	+++	++	++	++	++
	+++	+	+	++	++
Three weeks after treatments ceased (i.e., seven weeks post-weaning)					
Old house	++	+	+	+	+++
	+++	+	+++	+++	++
	++	+++	+	++	+++
New house	+++	+	+++	+++	+++
	+	+++	+++	+	++
	++	++	+	++	+

* Pig died (Rectal swab +++ immediately *post-mortem*).
The estimation of growth and haemolytic reaction on rectal swabs in this table and Table VII is given by:
+ Under 20 colonies per plate of non-haemolytic *E. coli*.
++ 20-200 colonies per plate of non-haemolytic *E. coli*.
+++ Confluent growth of haemolytic and non-haemolytic *E. coli*.

TABLE VI

THE EFFECT OF TREATMENTS DURING FOUR-WEEK POST-WEANING PERIOD ON PERFORMANCE OF PIGS SLAUGHTERED FOR BACTERIOLOGICAL EXAMINATION AFTER FOUR WEEKS

Treatment	Control	Lactic acid	Propionic acid	Calcium propionate	Calcium acrylate	Significance of differences	Standard error of difference	†Multiple range test	House		Significance of differences
									Old	New	
Treatment symbol	A	B	C	D	E						
Feed intake (lb. per day)	2·03	2·31	2·01	2·00	1·89	N.S.	0·176		1·89	2·21	*
Growth rate (lb. per day)	0·73	0·98	0·70	0·72	0·48	*	0·122	ECDAB	0·60	0·84	**
Feed utilisation (lb. feed per lb. gain)	2·90	2·41	2·89	2·85	4·09	**	0·445	BDCAE	3·24	2·82	N.S.

† Multiple range test (Duncan, 1955) was carried out where a significant "F" value obtained. The difference between any pair of treatments not underlined by the same line is significant at 5 per cent. level.

controls. This was particularly noticeable in the old house where duodenal counts for control pigs were 112 and 145 mill. organisms per ml. intestinal liquid, while the counts for controls in the new house were 2·70 and 0·40 mill. organisms per ml. Rectal swabs from control pigs also gave a confluent growth of both haemolytic and non-haemolytic *E. coli* while swabs from all treated pigs gave less than 200 colonies per plate of non-haemolytic *E. coli* only. Table V shows that three weeks after treatments had ceased rectal swabs giving confluent growth of both haemolytic and non-haemolytic *E. coli* were distributed randomly amongst all treatments and controls.

Growth and performance data for the four-week post-weaning period of pigs slaughtered in the bacteriological study are given in Table VI. Pigs on the lactic acid treatment grew significantly ($P<0·05$) more quickly than control pigs and pigs given the other treatments. The 34 per cent. faster growth rate of the lactic acid group than the untreated controls was due to a 13 per cent. greater feed intake and 17 per cent. better feed utilisation. Pigs fed calcium acrylate had poorer feed intake and significantly poorer ($P<0·05$) growth rate and feed utilisation than controls and all other treatments.

The house difference in *E. coli* count was reflected in the growth and performance of untreated control pigs which grew at 0·86 lb. per day in the new house and 0·61 lb. per day in the old house and also converted more efficiently. Treated pigs also grew more quickly and converted more efficiently in the new house in the bacteriological study.

Casualty Pigs

Bacteriological results of the three pigs which died during the treatment period are given in Table VII. Two of the pigs were in the growth trial and the other in the bacteriological study. Two pigs on the calcium acrylate treatment, numbers 322 and 34, died at 10 and 15 days post weaning respectively. Both these pigs had shown reluctance to accept the mixture of food, water and calcium acrylate.

TABLE VII
BACTERIOLOGICAL FLORA OF CASUALTY PIGS

Treatment	Pig No.	*E. coli* count (mill. org. per ml. liquor)		Rectal swabs	
		Duodenum	Jejunum	Growth	Density
Control	16	0·5	0·2	*E. coli*	+++
Calcium acrylate	322	2,800	30,600	*E. coli*	+++
Calcium acrylate	34	22,000	16,000	*E. coli* *Strep. faecalis*	+++

N.B. All *E. coli* isolated were haemolytic.

Discussion

Although all treatments were effective in controlling haemolytic *E. coli* and reducing non-haemolytic *E. coli* in the gut, these effects were apparent only during the period of application and ceased when treatments were withdrawn. Growth and performance of pigs on the different treatments were variable, but in both the growth trial and the bacteriological study pigs on the lactic acid treatment made more rapid and more efficient live-weight gains than control pigs and pigs given the other treatments. This was due, partly, to low feed intakes of pigs on some treatments, particularly calcium acrylate. This resulted from the unpalatability of some of the compounds used and as they were given in the drinking water on top of the food, their unacceptability was also reflected in lowered feed intake. Two pigs on the calcium acrylate treatment died, having high counts of haemolytic *E. coli* in the gut, but samples taken from pigs on this treatment in the bacteriological study yielded no haemolytic *E. coli* and only low counts of non-haemolytic *E. coli*. This apparent anomaly was probably due to the pigs which died finding calcium acrylate unacceptable, although in cases where it was eaten it was effective.

The beneficial effects of lactic acid in the control of *E. coli* in the gut and in improving the performance of pigs agrees with the findings of Burnett and Hanna (1963) and Kershaw *et al.*, (1966) and similar results have been obtained by introducing *Lactobacillus acidolphus* into the diet of pigs (Redmond & Moore, 1963). The mode of action of lactic acid is not clear, but in the gut it is produced by *Lactobacillus* sp. and Leitgeb (1961) has shown a strong negative correlation ($r = -0·626$) between the count of *Lactobacilli* (mainly *Lactobacillus bifidus*) and the count of *E. coli* in pigs. The presence of large numbers of *Lactobacilli* may result in the production of sufficient lactic acid to provide an environment unsuitable for *E. coli* and the same effect may be achieved by giving lactic acid orally. An alternative suggestion is that feeding lactic acid to the pig may result in an environment suitable for the existence and rapid multiplication of *Lactobacilli* which may in turn produce a substance capable controlling *E. coli*. Some strains of *Lactobacillus* (notably *L. acidolphus*) have been reported to produce such a substance called lactocidin (Vincint, Veomatt & Riley, 1959).

The use of a 1 per cent. solution of 80 per cent. lactic acid cost 3s. 6d. per pig, at current prices, for the four-week period and this would have been offset by a saving of 1s. 4d. per pig due to improved feed utilisation in the growth trial. However, better feed utilisation of the lactic acid fed pigs would have resulted in a saving of 3s. 4d. per pig using the growth data of the bacteriological study, and using the data of an earlier experiment (Kershaw *et al.*, 1966) there would have been a saving of 4s. per pig. Thus, at the level used, lactic acid would not be justified economically, solely on the basis of its growth promoting properties. The decision to use 0·8 per cent. lactic acid was an arbitrary one, and further work is needed to study the effects of lower levels of inclusion for longer periods on growth and feed utilisation.

The use of a new house to simulate good husbandry conditions provided an environment which would be an extreme under normal practical conditions, while the old house was left in such a condition that it was below average practical standards. Although, in the bacteriological study, pigs in the new house had lower

(*Concluded at foot of col. 1 overleaf*)

Fitting a Prosthetic Crown to a Canine Tooth in a Police Dog

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Vet. Rec. (1968). 83. 464-465

Introduction

BELOW is described the fitting of a chrome cobalt steel alloy crown to the fractured canine tooth of an Alsatian police dog. There appears to be no literature available on the fitting of a prosthesis on such a weight-bearing tooth.

History

The two years and 11 month old male Alsatian dog was presented at surgery with a fractured lower left canine tooth. The tooth had been snapped off some eight months previously when it had become entangled in his choker chain. No treatment had been sought at the time because it was believed that conservative treatment was impossible, and that extraction of the stump would so reduce his gripping capacity that compulsory retirement from duty would be necessary. Police handlers commented that his bite was still the strongest of the local dogs in training.

Clinical Examination and Treatment

About one-third of the crown remained. This was grossly discoloured, but there was no evidence of pain on examination. The pulp cavity had been exposed.

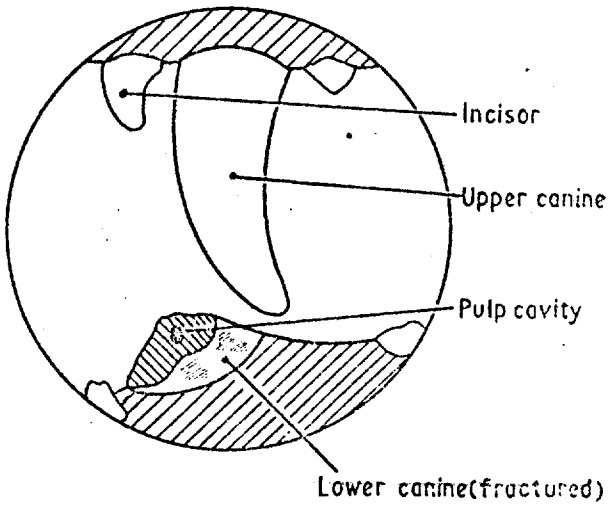

FIG. 1.

Mr. W. G. Dobbs, L.D.S., B.D.S. was contacted and it was decided that a chrome cobalt steel alloy crown might be fitted. Since the dog was somewhat difficult to handle it was also decided that he should be anaesthetised at our surgery and delivered to the dental surgery in a relatively easily manageable state.

Operation

The fitting of the prosthesis was somewhat experimental. Three attempts, with modifications to technique, were made before success was achieved.

The dog's weight was estimated at 95 lb. He was premedicated with 2 c.c. Promazine hydrochloride (Sparine-Wyeth) intramuscularly, followed half an hour later by 13 c.c. Pentobarbitone sodium (Sagatal, M. & B.) intravenously for anaesthesia.

An attempt was made to radiograph the remains of the tooth using a "Kingsway" dental X-ray machine. Due to its size and the size of the dental film available, it was found impossible to show the root in its entirety. The radiograph confirmed, however, that the pulp cavity had been exposed for the eight months (see Fig. 2).

In preparing the stump the pulp cavity was first excavated—a digging rather than drilling operation since the contents were found to be necrotic. Every attempt was made to remove all the debris from the cavity. Overhangs were removed from the stump by drilling, and finally two grooves were drilled along the axis of the stump on its medial and lateral

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The Effect of Lactic Acid.—Concluded.

counts of *E. coli* in the gut and better growth performance during the treatment period, this was not the case in the growth trial where pigs in the new house grew more slowly than pigs in the old house. However, in the eight-week post treatment period pigs in the new house had significantly better feed utilisation. No explanation for this difference in performance could be found.

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ESTIMATING GASTROSPLENIC BLOOD FLOW AND VOLATILE FATTY ACID ABSORPTION FROM THE FORESTOMACHS OF CALVES

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SUMMARY

Blood flow from the coeliac artery, which is distributed to the rumen, reticulum, omasum, abomasum, and the spleen, and eventually passes through the gastrosplenic vein, was estimated in seven calves anesthetized with nembutal. The abdominal cavity was entered through an incision along the line of the 12th rib. Erythrocytes tagged with P^{32} served as markers, and the isotope dilution method was used to determine the rate of blood flow. The tagged erythrocytes were injected into the coeliac artery and collected from the gastrosplenic vein. The first radioactivity appeared in the gastrosplenic vein 1 to 2 sec. after the injection, and the maximum concentration occurred in 12.4 sec. The average time required for all the injected, labelled erythrocytes to pass through the gastrosplenic vein was 31.3 sec. The mean rate of gastric blood flow was calculated to be 764 ml. per minute per 100 lb. of body weight. Based on the analysis of serial blood samples obtained from a calf fitted with a polyethylene catheter into the gastrosplenic vein, it was calculated that approximately 63 g. of acetic and 25 g. of propionic acid were absorbed for each pound of dry feed (a 2:1 mixture of alfalfa hay and grain concentrate) consumed during a 24-hr. period.

The measurement of gastric blood flow is of particular interest in dairy cattle digestion studies. The ultimate aim is to measure quantitatively the nutrients and end-products of digestion absorbed from the forestomachs in intact animals. Measurements made on sheep using copper-constantan thermocouples showed that blood flowed through the portal vein at the rate of approximately one liter per minute *(4)*. Portal blood analyses have been used to study absorption of ruminal fermentation products *(1)*.

In this study, an estimate was made of the blood flow from the coeliac artery, which is distributed to the rumen, reticulum, omasum, a part of the abomasum, and the spleen, and eventually passes through the gastrosplenic vein. Since the gastric blood flow of cattle represents an isolated system of blood vessels, excepting the arterial blood to the liver, it is especially well adapted to measure blood-flow rate. Schematic diagrams of gastric arteries and the portal system of cattle are shown in Figures 597 and 602a of Sisson and Grossman *(12)*.

EXPERIMENTAL PROCEDURES AND RESULTS

Seven dairy calves, 4–7 mo. of age, anesthetized with nembutal, were used. Erythrocytes tagged with P^{32} served as the marker and the isotope dilution method was used to determine the rate of blood flow. Tagged erythrocytes were injected into the coeliac artery and collected from the gastrosplenic vein which drains the stomachs and spleen.

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The abdominal cavity was entered through the right side by an incision along the line of the 12th rib. First, the periosteum was reflected from the lateral surfaces of sections of the 11th, 12th, and 13th ribs and the bones removed, leaving the medial layers intact. Removal of parts of the 11th and 13th ribs in the manner described facilitated the anterio-lateral movement of the liver without penetrating the thoracic cavity. The junction of the gastrosplenic vein and the portal vein was exposed by blunt dissection at the anterior end of the pancreas. The hepatic artery, which lies a few millimeters dorsal to the gastrosplenic vein and approximately 3 cm. anterior to the mesenteric artery, was also exposed by blunt dissection. This artery was catheterized through a California bleeding needle with polyethylene tubing (P.E. 90). The catheter was moved through the hepatic artery to the junction with the coeliac artery. The gastrosplenic vein was then cannulated with a 12-gauge needle, in preparation for taking serial blood samples.

The cells of 10 ml. of whole blood were labelled in vitro with 200 μc. of Phosphorus^{32}. The cells were separated by centrifugation, washed, and suspended in cold saline *(7)*. Three ml. of the tagged cells were injected instantaneously into the coeliac artery via the polyethylene catheter, using a 5-cc. syringe. The hepatic artery was temporarily occluded during dosing by finger pressure at a point ventral to the insertion of the catheter. Blood samples from the gastrosplenic vein were obtained at approximately 2-sec. intervals for the first 60 sec. after dosing, and at less frequent intervals thereafter.

The blood flow was determined by the dilution method *(6)*, using the following formula:

$$\text{Blood flow in milliliters per minute per 100 lb. body weight} = \frac{a}{v} \times \frac{60}{t} \times \frac{100}{w}.$$

In the formula, a equals counts per minute injected into coeliac artery, v equals average counts per minute per milliliter of gastrosplenic blood for time t, t equals time required for all of the radioactive cells to pass through the gastrosplenic vein had there been no recirculation, and w equals the body weight in pounds. Total blood volume was determined by the formula of Hansard *et al.* *(7)*.

Radioactivity was determined in the gastrosplenic blood and the counts per minute plotted in relation to time after dosing. The tagged cells appeared in the gastrosplenic vein along a rising and falling curve (as shown in Figure 1). One to two seconds after injection the first tagged cells appeared and reached a maximum concentration at approximately 12 sec. By disregarding recirculation and extrapolating the descending curve, as indicated by the broken line in Figure 1, it was calculated that the average time required for all of the injected, labelled erythrocytes to pass through the gastrosplenic vein was 31.3 sec. The mean rate of gastric blood flow was calculated to be 764 ml. per minute per 100 lb. of body weight. Results are summarized (Table 1).

During the early determinations, unusually low values were obtained with two calves. Both calves were determined to have had low blood volumes at the time of the blood flow measurements, presumably because of losses during surgery. In one of these calves, blood was not given for replacement and the

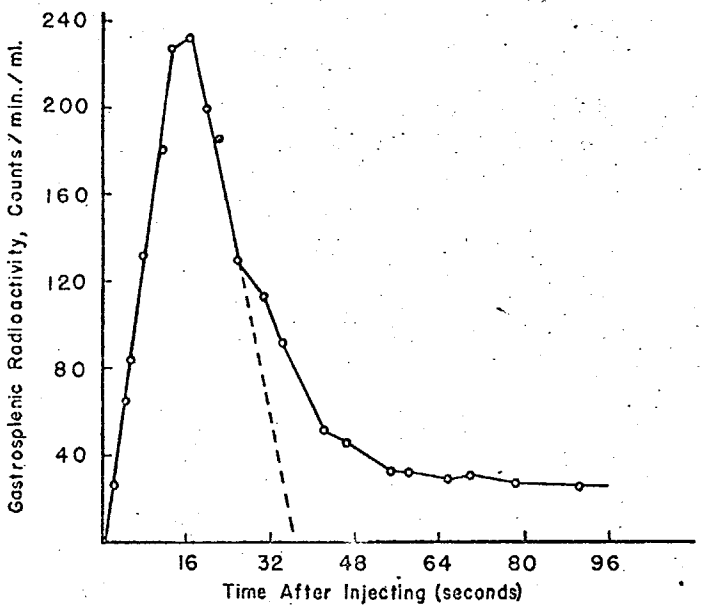

FIG. 1. Radioactivity curve of Calf 412, showing the appearance of tagged erythrocytes in blood drawn from the gastrosplenic vein, in relation to time after instantaneously injecting in the coeliac artery. Broken line shows method of extrapolating, to estimate area under curve had there been no recirculation.

TABLE 1

Time and rate of blood flow through the forestomachs of calves, measured between the hepatic junction of the coeliac artery and the gastrosplenic vein, using erythrocytes tagged with Phosphorus[32]

Calf No.	180	189	189	254	254	411	412	244	Mean
Age *(mo.)*	5	6	6	4	4	7	7	5	
Trial No.	1	1	2	1	2	1	1	1	
Time after injection required to reach maximum radioactivity in the gastrosplenic vein *(sec.)*	12	13	11	13	12	12	15	11	12.4
Time required for all tagged erythrocytes to pass through the gastrosplenic vein *(sec.)*	29	37	36	30	24	24	36	34	31.3
Blood flow *(ml/min/100 lb. body wt.)*	780.2	872.2	856.1	865.9	878.1	741.1	365.0	751.0	763.7
Total blood volume *(% body wt.)*	[a]	6.6	6.6	6.6	6.6	5.7	5.1	[a]	6.0[b]
Transfused blood administered during surgery *(ml/100 lb. body wt.)*	110	205	205	360	360	220	300	340	300

[a] Blood volume not determined.
[b] Blood volume value from one trial only included in the mean.

data were not included. In the other calf, even though blood loses were partly replaced, the blood volume was only 5.1% of the body weight. The data from this calf, No. 412, were included in Table 1. The data on these calves and the others with blood volumes in the normal range (Table 1), suggest a direct relationship of blood flow rate to per cent blood volume.

Serial blood samples of gastrosplenic blood were obtained from three other calves to test the feasibility of applying gastrosplenic blood flow to volatile fatty acid absorption. The blood was obtained at various intervals for 24 hr. through a polyethylene catheter placed in the gastrosplenic vein by surgical procedure. Samples of jugular blood were taken periodically also, to obtain an estimate of the amount of volatile fatty acids in general circulation, and these values were used to correct for arterial volatile fatty acids. The blood samples were analyzed for volatile fatty acids by chromatographic procedures *(8)*, after making protein-free filtrates and steam-distilling. The difference in volatile fatty acid content between gastrosplenic and jugular blood was considered to be the amount absorbed. The diurnal changes in the volatile fatty acids of gastrosplenic blood, corrected for circulating volatile fatty acids in arterial blood, are shown

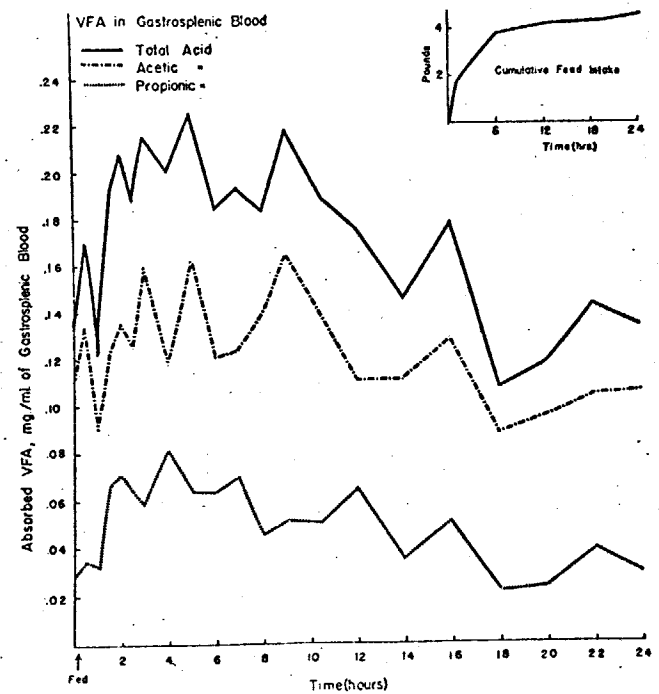

FIG. 2. The concentration of volatile fatty acids (acetic, propionic, and traces of butyric) in the gastrosplenic blood corrected for arterial VFA, and the cumulative feed intake of a 5-mo.-old Jersey calf during the 24-hr. period after allowing it free access to a fresh feed of high roughage pellets (two parts alfalfa and one part grain).

(Figure 2) for the calf, along with its cumulative feed intake. This calf consumed 4.3–4.6 lb. of feed per day for three days previous to the test, and 4.5 lb. during the period of the test.

Using the average blood-flow rate in Table 1, obtained from the anesthetized calves, and the average amount of acetic and propionic acid absorbed, it was calculated (as shown in Table 2) that an average of 63 g. of acetic and 25 g. of propionic acid were absorbed for each pound of dry feed (a 2:1 mixture of alfalfa hay and grain concentrate) consumed by this calf during the 24-hr. period. Results for two other calves, fed a 4:1 ratio of alfalfa hay and grain concentrates, also are given (Table 2).

TABLE 2

Absorbed volatile fatty acids in the gastrosplenic blood of three different calves fed pelleted rations containing alfalfa hay, ground corn, and oats

Calf No.	Age	Hay:grain ratio	Body weight	Dry feed eaten	Absorbed VFA[a]	
					Acetic[b]	Propionic[b]
	(mo.)		(lb.)		(g.)	
J-1238	5	2:1	175	3.82	62.8	25.3
G-1262	5	4:1	172	4.22	50.8	12.1
H-1265	4	4:1	295	8.80	48.3	16.9

[a] Net VFA after correcting for VFA in general circulation.
[b] Grams of VFA absorbed for each pound of dry feed consumed during 24 hr. Average moisture content of feed was 14%.

In separate digestion trials, the digested organic matter was determined to be 58 and 55%, respectively, of the 2:1 and 4:1 mixtures, using bull calves 4 to 5 mo. of age, which were consuming 4–8 lb. of the pelleted ration daily.

DISCUSSION OF RESULTS

The uniformity of blood-flow rates obtained (Table 1) demonstrate the usefulness of radioactive-tagged erythrocytes for determining organ blood flow, the only exception being Calf 412, which also was determined to be low in blood volume. The isotope dilution method has the advantage of being relatively inexpensive in laboratories where equipment is available for measuring radioactivity. In the direct application of the gastric blood-flow rates obtained, it is important to consider that ruminal blood flow may be different in the intact, conscious animal. Anesthesia induced with nembutal slows down rumen motility *(5)*. The effect of nembutal on gastrosplenic blood flow has not been measured. Also, it has been proposed that absorption of volatile fatty acids partly is regulated by nerve action *(1)*. This also may cause changes in the rate of gastric blood flow. Gastric blood flow may also vary significantly with time after feeding.

Since it is known that the volatile fatty acids produced by rumen fermentation are absorbed in calves previous to the passage of rumen digesta to the abomasum *(3)*, these data have further useful application in estimating the amount of total feed energy absorbed as volatile fatty acids in the calf's forestomach. This can be illustrated by applying the energy values given by Brody *(2)* for hay, grain, and acetic and propionic acids. The amount of energy absorbed as volatile fatty acids was calculated to be 29% of the total calories in the digested organic matter of the 2:1 mixture. An undetermined amount of useful energy present in the gastrosplenic blood in the form of butyrate metabolites, resulting from the metabolism of butyric acid in the rumen wall *(10)*, was not considered in the calculations.

Only traces of butyric and higher acids were found in the gastrosplenic blood of the catheterized calves. The following ranges in proportion of volatile fatty acids in the gastrosplenic blood from five other calves used in this experiment were found: acetic, 78–92, propionic, 8–21, and butyric, 0–2%. This is less butyric acid than was reported by Phillipson *(11)* for the ruminal blood of sheep. In our experiment, it seems reasonable to assume that absorbed butyric acid was metabolized by the rumen epithelium to other energy compounds based on the findings of Pennington *(10)*. In present studies, attempts are being made to measure the butyrate metabolites.

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GASTRIC MUCOSAL INJURY BY FATTY AND ACETYLSALICYLIC ACIDS

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Ions and water soluble substances move slowly across the nonsecreting gastric mucosa,[1] but compounds that are fat soluble cross it rapidly. Well established principles of general physiology are that cell membranes behave as if they were a mosaic of protein and fat and that fat soluble substances diffuse rapidly through a cell membrane which water soluble substances fail to penetrate. Hogben *et al.*[2] have demonstrated in detail that drugs that are weak electrolytes may be quickly absorbed from acid gastric contents if they are unionized and fat soluble in acid solution. For example, at pH 2 acetylsalicylic acid (pK 3.5) is more than 95 per cent in the unionized, fat soluble form, and it diffuses across the mucosa. At pH 7 it is completely ionized and fat insoluble, and it is poorly absorbed from the stomach. Similarly, acetic acid, but not acetate, is absorbed more rapidly than HCl.[3]

The work reported here shows that when compounds in their fat soluble form diffuse through the mucosa they can change its characteristics. A mucosa through which acetic, propionic, butyric, or acetylsalicylic acid is diffusing develops increased fluxes of hydrogen, sodium, potassium, and chloride ions, and the increases persist after the compounds are removed. The changes are chemical signs of injury, and desquamation and bleeding may accompany them.

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Materials and Methods

Four dogs with vagally denervated pouches of the oxyntic gland area of the stomach prepared by Code were used. The animals were in good health, accustomed to the laboratory and trained to stand quietly during the tests. The opening of the pouch in the midline of the abdominal wall was fitted with a Vitallium cannula which did not leak. Each pouch could be filled with 40 ml. of fluid without the animal's showing signs of discomfort. Before each test the dogs were deprived of food and water for 36 hours, and the pouches showed only small spontaneous secretion during control periods.

One end of a polyethylene tube was inserted into the cannula, and the other end was attached to a metal 3-way stopcock which in turn was attached to a 50 ml. syringe held vertically about 10 cm. above the level of the cannula. A second syringe was connected to the other opening of the stopcock. At the beginning of the experimental period the pouch was washed clean with 30 ml. aliquots of the appropriate wash solution, and the last of the wash was removed by gentle suction, leaving a residual volume. The barrel of the vertical syringe was removed, and the stopcock was closed. Forty milliliters of the test solution were pipetted into the syringe, quickly forced into the pouch, and thoroughly mixed with the residual volume. Then an approximately 10-ml. sample was withdrawn, measured to 0.1 ml., and saved for analysis. The instant of removing the sample was counted the beginning of a 30-minute period of observation. During the period the contents of the

pouch were frequently mixed, either by spontaneous contractions or by withdrawing the barrel of the syringe. At the end of the period the contents were withdrawn, again leaving a residual volume. The 30-minute sample was measured to 0.1 ml. and saved for analysis. If the composition of the test solution was not to be changed for the next period, 40 ml. were pipetted into the syringe, mixed in the pouch, and sampled. If the composition was to be changed, the pouch and tubing were quickly washed with 4 20-ml. samples of the wash solution. These washings were combined in a 100-ml. flask, made up to volume, and saved for analysis. Then the pouch was filled with 40 ml. of the new test solution which was mixed and sampled.

The test solutions are described under *Results*. When the contents of the pouch were to be acid in the next period an acid wash solution was used containing 15 mN of NaCl, 100 mN of HCl, and 78 mN of mannitol. When the contents were to be neutral the wash solution used contained 15 mN of NaCl and 250 mN of glycine.

Na^{24} was counted in a deep well γ-ray scintillation counter which was standardized every day, and the counts were corrected for decay to an arbitrary zero time. Sodium and potassium were measured with a direct reading flame photometer, and each sample was bracketed with appropriate standards. Chloride was measured with an Aminco Cotlove titrator. Total acidity of the HCl solutions was measured by titration to the phenolphthalein end point, and pH was measured with a glass electrode. Total acid in the glycine solutions was calculated from the pH and a previously determined titration curve. D_2O was measured with a Consolidated Engineering mass spectrometer.

Residual volume at the beginning of a period was calculated from the dilution of Na^{24} in the initial sample as compared with its concentration in the test solution. Residual volume at the end of a period was calculated as the volume of the final sample which would contain the Na^{24} found in the subsequent wash. When no calculation of residual volume was possible the mean of the day's measurements was used. Volume of fluid present in the pouch at zero time was taken to be 40 ml. plus residual volume minus initial sample, and volume at the end was taken to be the sample recovered plus residual volume. Residual volumes for the 4 dogs ranged from 3.5 ± S.D. 0.8 to 3.9 ± S.D. 0.9 ml. Because other volumes were measured to 0.1 ml. the error of estimate of volumes in the pouches averaged 3 per cent.

The quantities of sodium, potassium, chloride, acid, and water entering or leaving in 30 minutes were calculated as the difference between the masses initially and finally present. By convention, net losses (absorption) or one way fluxes from pouch to animal (insorption) are reported as negative quantities, and net gains (enterosorption) or one way fluxes from animal to pouch (exsorption) as positive ones. Flux of sodium from fluid to animal (insorption) was calculated as the difference between total counts of Na^{24} initially and finally present divided by the mean specific activity of sodium in the fluid throughout the period. In 11 of 90 periods when the sodium flux was very small, physically impossible positive values for insorption were obtained: these reflect the cumulative errors of the several measurements used in the calculation. All values for electrolytes are reported as micromoles per 30 minutes. Flux of water from pouch to animal (insorption) was calculated by subtracting from the product of the initial volume and its moles per 100 ml. excess D_2O the product of the final volume and its moles per 100 ml. excess D_2O and dividing the result by the mean moles per 100 ml. excess D_2O. Insorption of water is reported as a negative number with units of milliliters per 30 minutes.

Results

Results obtained when the pouches were irrigated with acid test solutions are given in table 1.

Each experiment was begun with a 30-minute control period in which the test solution used contained 100 mN of HCl, 15 mN of NaCl, 78 mN of mannitol, 0.2 μc. of Na^{24} per 100 ml. and 3.6 moles per 100 ml. excess D_2O. (The dogs were used concurrently for other experiments to be reported elsewhere, and each of these also began with an identical control period. All control data from both series were pooled

TABLE 1

Movement of electrolytes and water across canine gastric mucosa; acid test solutions

Electrolyte values in microequivalents per 30 minutes; water in milliliters per 30 minutes, both ± S.E. Positive values are gains by pouch contents; negative values are losses. For Na and H_2O "in" designates insorption.

Solutions	n	H^+, net	Na, net	Na, in	K, net	Cl, net	H_2O, net	H_2O, in
HCl controls[a]	38	−100 ±17	178 ±10	−18 ±4	9 ±1	104 ±34	1.4 ±0.2	−14.2 ±1.7
Acetic[b]	6	−723 ±109	626 ±60	−65 ±16	48 ±5	746 ±115	2.6 ±0.9	−14.4 ±1.7
HCl postacetic[a]	6	−431 ±19	737 ±131	−44 ±13	38 ±4	244 ±108	3.1 ±0.9	−10.8 ±2.9
Propionic[b]	4	−1069 ±38	692 ±46	−75 ±31	31 ±5	561 ±58	1.5 ±0.7	−15.1 ±1.4
HCl postpropionic[a]	4	−376 ±43	587 ±109	−17 ±3	18 ±7	46 ±117	2.5 ±0.8	−10.9 ±0.9
Butyric[b]	4	−1005 ±59	700 ±120	−71 ±25	43 ±4	677 ±61	2.6 ±1.0	−14.1 ±0.5
HCl postbutyric[a]	4	−545 ±193	574 ±193	−40 ±14	28 ±5	22 ±92	2.1 ±0.6	−14.1 ±0.5

[a] 100 mx of HCl, 15 mx of NaCl, 78 mx of mannitol.

[b] 100 mx acetic (or propionic or butyric) acid, 15 mx of NaCl, 78 mx of mannitol.

and are reported here.) The results in the first line of table 1 show a small loss of H^+ equal to 3 per cent of that in the pouch. Gain of Na^+ was about equally divided between that exchanging for H^+ and that accompanying Cl^- into the lumen. Insorption of Na^+ equal to 3 per cent of that in the pouch occurred.

The control period was followed by 2 30-minute periods in which the acid test solution contained 100 mx acetic (or propionic or butyric) acid, 15 mx of NaCl, 78 mx of mannitol, 0.2 μc. Na^{24} per 100 ml. and 3.6 moles per 100 ml. excess D_2O. No systematic differences were found between the first and second of these periods, and the results were pooled. The experiment was concluded by 2 more 30-minute periods in which the original HCl test solution was again used. The results for these 2 periods were also pooled. Three experiments were done with acetic acid and 2 with each of the other acids.

When organic acid was present between 22 and 32 per cent of it disappeared in 30 minutes, but it was not exchanged for Na^+, doubtless because it crossed the mucosa in the unionized form. There was, however, outpouring of Na^+ accompanied by Cl^-. Both Na^+ insorption and net K^+ movement increased 4-fold. If the net gain of Na^+ is divided by the net change in volume, a concentration much higher than that of Na^+ in extracellular fluid is obtained; therefore, it is unlikely that the net gains can be accounted for by simple transudation. All changes indicate that the mucosal barriers were partially disrupted by the organic acids.

Mucosal changes produced by the organic acids persisted for at least an hour, for during the subsequent 2 periods in which the HCl test solution was used the loss of H^+ from the lumen was much greater than in the control period. This H^+ was exchanged for Na^+, and in addition Na^+ moved into the lumen accompanied by Cl^-. K^+ movement and Na^+ insorption remained elevated. In the final periods blood appeared in the test solutions.

After a 30-minute control period in which the acid HCl test solution was used the pouches were irrigated with neutral test solutions containing 100 mx of acetic (or propionic or butyric) acid neutralized with trishydroxyaminomethane (TRIS), 15 mx of NaCl, 78 mx of glycine and 0.2 μc. of Na^{24} per 100 ml. These solutions were

strongly buffered below pH 5 and above pH 7 and weakly buffered between these limits. Their pH was always above 5.5, 2 units above the pK's of the acids, and it is in this sense that they are called neutral. As controls for these periods we present data in the first line of table 2 obtained when the pouches were irrigated with a neutral solution containing 250 mN of glycine, 15 mN of NaCl, and 0.2 μc. of Na^{24} per 100 ml. When the neutral solution had been removed from the pouch each experiment was terminated with 2 30-minute periods in which the HCl test solution was again used. Data obtained in each pair of periods was pooled. The results in table 2 show that the neutral solutions had no effect upon the movement of water and electrolytes across the mucosa.

A series of experiments in which the pouch of 1 dog was irrigated with solutions containing acetylsalicylic acid are described in table 3. In the first 30-minute period of the experiment of May 21, the pouch was filled with the acid test solution described above. In the next 2 periods the pouch was irrigated with a similar solution of 100 mN of HCl to which 20 mM of acetylsalicylic acid had been added. The aspirin was in solution, not in suspension. It remained in solution while being instilled into the pouch. Finally, in 2 more periods, the original test solution was used. During the periods of irrigation with acetylsalicylic acid there were very greatly increased fluxes of all ions; bleeding began, and there was large enterosorption of water. The changes persisted after removal of the acetylsalicylic acid, and they are sign of severe disruption of the mucosal barriers.

On the fourth day after this test the pouch bled copiously during the preparative washing, and bleeding continued during the first test period in which the acid test solution was used. Nevertheless, during the next 2 periods in which the test solution consisted of 20 mM of acetylsalicylic acid exactly neutralized with TRIS, 15 mN of NaCl, and 250 mN of glycine, bleeding and enterosorption of sodium and chloride diminished. This solution was well buffered below pH 4.5, and the measured pH of

TABLE 2

Movement of electrolytes and water across canine gastric mucosa; neutral test solutions

Electrolyte values in microequivalents per 30 minutes; water in milliliters per 30 minutes, both ± S.E. Positive values are gains by pouch contents; negative values are losses. For Na "in" designates insorption.

Solutions	n	H^+, net	Na, net	Na, in	K, net	Cl,[d] net	H_2O, net
Glycine controls[a]	20	56	153	−32	17	175	1.0
		±17	±7	±4	±2		±0.2
Acetate[b]	4		49	−39	21	409	1.1
			±43	±8	±2	±66	±0.4
HCl postacetate[c]	4	99	79	−5	8	109	1.3
		±21	±4	±4	±2	±26	±0.2
Propionate[b]	4		161	−24	6	261	0.4
			±44	±13	±2	±63	±0.3
HCl postpropionate[c]	4	−80	184	0	4	181	1.9
		±49	±11	±5	±2	±97	±0.7
Butyrate[b]	4		193	−35	12	244	0.2
			±15	±4	±2	±25	±0.4
HCl postbutyrate[c]	4	86	162	−2	9	194	1.9
		±116	±47	±9	±1	±65	±0.3

[a] 250 mN of glycine, 15 mN of NaCl.

[b] 100 mN of acetic (or propionic or butyric) acid neutralized with TRIS, 15 mN of NaCl, 78 mN of glycine.

[c] 100 mN of HCl, 15 mN of NaCl, 78 mN of mannitol.

[d] Number of observations less than n given.

TABLE 3

Effect of acetylsalicylic acid in acid and neutral solutions on movement of water and electrolytes across gastric mucosa; dog X-928

Electrolyte values in microequivalents per 30 minutes, water in milliliter per 30 minutes. Positive values are gains by pouch contents; negative values are losses. For Na "in" designates insorption.

30	Solution	H^+, net	Na, net	Na, in	K, net	Cl, net	H_2O, net	Blood[e]
May 21, 1963; acetylsalicylic acid in acid solution (ASA-HCl)								
1	HCl[a]	−158	263	3[d]	6	87	1.3	0
2	ASA-HCl[b]	−1046	1685	−90	101	624	8.7	+
3	ASA-HCl[b]	−577	2854	−129	136	1886	17.7	++
4	HCl[a]	−531	2614	−188	109	1699	14.6	+++
5	HCl[a]	2	2047	−59	84	1523	12.5	++++
May 24, 1963; acetylsalicylic acid in neutral solution (ASA-TRIS)								
1	HCl[a]	22	867	−22	37	681	6.2	++++
2	ASA-TRIS[c]		598	−73	25	545	1.8	++
3	ASA-TRIS[c]		337	−59	19	378	0.5	+
4	HCl[a]	−219	931	−47	39	391	5.2	+++
5	HCl[a]	−181	1015	−26	46	611	6.4	++++
June 13, 1963; acetylsalicylic acid in neutral solution (ASA-TRIS)								
1	HCl[a]	−213	299	−4	10	42	0.9	0
2	ASA-TRIS[c]		276	−22	13	275	0.3	0
3	ASA-TRIS		264	−28	14	240	0.7	0
4	HCl[a]	−303	320	−28	15	−17	0.1	0
5	HCl[a]	59	560	11[d]	19	341	3.9	+

[a] 100 mx of HCl, 15 mx of NaCl, 78 mx of mannitol.

[b] 20 mx of aceytlsalicylic acid, 100 mx of HCl, 15 mx of NaCl, 78 mx of mannitol.

[c] 20 mx of aceytlsalicylic acid exactly neutralized with TRIS, 15 mx of NaCl, 250 mx of glycine.

[d] Physically impossible positive value not different from zero probably resulting from error in volume measurement.

[e] + designates faintest detectable tinge of blood; ++++ designates "coffee grounds."

the samples was between 4.8 and 5.3. It is clear that the acetylsalicylic acid in neutral solution did not harm the mucosa.

The dog was allowed to rest until June 13 by which date its pouch had apparently healed. Then irrigation with neutralized acetylsalicylic acid had no effect upon water or electrolyte movement across the mucosa.

Experiments with acetylsalicylic acid in acid and neutral solutions were performed on 2 other dogs with similar, but not so spectacular, results.

Discussion

There is nothing new in this demonstration that simple organic acids and acetylsalicylic acid can damage the gastric mucosa. Teorell[3] observed that acetic acid "inflames" the mucosa, and there is a large literature on the effects of acetylsalicylic acid on the stomach.[4,5] Nor is the observation that fat soluble substances can penetrate the mucosa novel. The results do show, however, that it is the combination of potential toxicity with conditions making penetration possible which determine that damage occurs. The agents are utterly innocuous in contact with the mucosa, but they become toxic when they are able to move into it. For the substances studied here, fat solubility is the determinant of their ability to penetrate. Once in or through the mucosal barrier the toxic compounds

damage it, perhaps by interfering with the underlying metabolic processes that maintain the barrier. Acetic, propionic, and butyric acids may act simply by rapidly carrying into the cells hydrogen ions that overwhelm the local buffers. Acetylsalicylic acid may be a protein precipitator or enzyme inhibitor. Other substances, such as fluoride and mersalyl, which also break the mucosal barrier,[6] may combine with specific components of the membrane or its supporting structures.

Damage to the mucosa can be characterized in chemical as well as in morphologic terms. Chemical damage alters the mucosal barriers, allowing increased fluxes of ions across the mucosa. The normal mucosa is divided into several compartments by barriers having different degrees of permeability. In figure 1 are shown the compartments and barriers based upon analyses of the canine gastric mucosa and supported by studies of the frog gastric mucosa.[7-10]

Erythrocytes, which in the dog are high in sodium and low in potassium, are confined within blood vessels, and there are 7 to 13 ml. of them in the smaller vessels of 1 kg. of mucosa. The plasma content of the mucosa, measured by radioiodinated serum albumin (RISA), is 54 to 78 ml. per kilogram, which means that 4 to 12 ml. of plasma accompany each milliliter of erythrocytes. Some of this plasma within the gastric mucosa may, however, be extravascular as it is in dog ileum.[11] When the mucosa becomes edematous as the result of partial venous occlusion the concentration of plasma protein in the mucosa is increased, and the space reached by RISA, calculated by dividing the quantity of RISA in 1 kg. of mucosa by its concentration in plasma, may be as great as 250 ml. per kilogram. The conclusion that a fraction of plasma protein readily crosses gastric capillary walls is supported by the observations that plasma albumin is a normal component of gastric juice[12, 13] and that about 4 per cent of the total daily metabolic degradation of intravenously administered RISA occurs in the stomach.[14] The space assigned to albumin in figure 1 was calculated on the assumption that the concentration of albumin in its extravascular compartment is the same as its concentration in plasma. If, as is much more likely, the extravascular concentration of albumin is lower than the plasma concentration, the extravascular

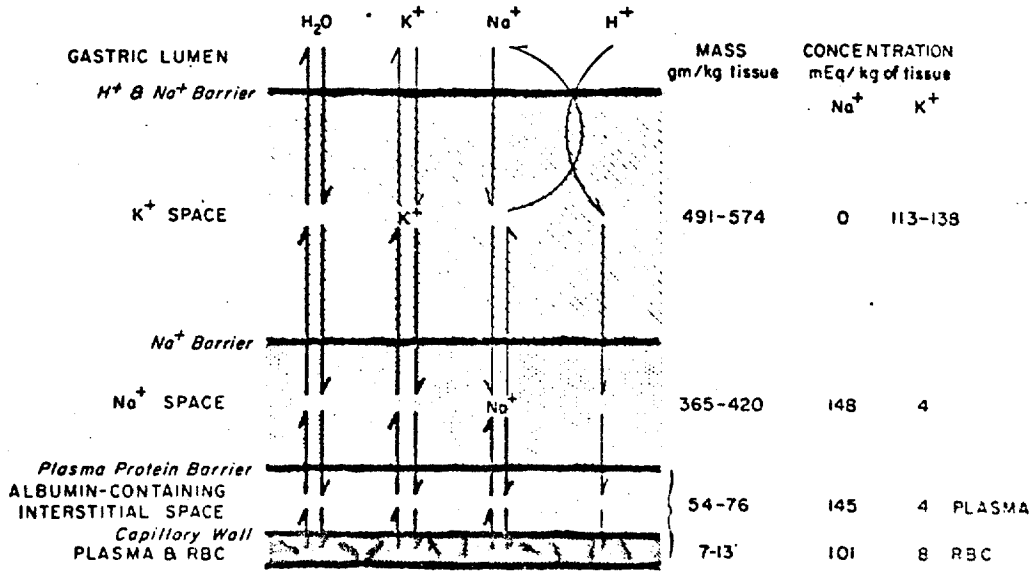

FIG. 1. Electrolyte distribution and barriers of the canine gastric mucosa. See text for references and for discussion of the assumptions upon which the calculations are based. The figure is purely schematic, and it is not meant to imply that all movement from blood to lumen need be through the potassium space.

space it occupies is correspondingly larger. If all extravascular sodium is extracellular, the extravascular space occupied by albumin and sodium may be coextensive.

The concentration of sodium is much higher in the mucosa than in skeletal muscle. When the quantity of sodium present in erythrocytes and plasma contained in 1 kg. of mucosa is subtracted from the total, a mass of 54 to 62 mEq. per kilogram remains. On the assumption that the concentration of the remaining sodium in extravascular space is the same (after a small Donnan correction) as in plasma water, the space reached by sodium is calculated to be 365 to 420 ml. per kilogram. There is no conclusive evidence whether this extravascular sodium is partially intracellular. The spaces containing sodium, erythrocytes, plasma, and extravascular space together contain 320 to 500 ml. of water. The whole mucosa contains 776 ml. of water per 1 kg., and if all of the sodium is extracellular only 276 to 456 ml. of water can be intracellular. This seems an unlikely distribution of tissue water. Ninety-five per cent of sodium in the mucosa equilibrates very rapidly with radioactive sodium in the plasma, and consequently, the barriers between plasma and all major sodium containing compartments must be freely permeable to the ion. On the other hand, sodium does not easily reach these compartments from the mucosal surface.

Potassium is the chief cation contained in that fraction of tissue water not reached by sodium, and most of it is doubtless intracellular. In constructing figure 1 I have assumed, perhaps incorrectly, that the potassium concentration in all extravascular space which also contains sodium is equal to the potassium concentration in plasma water. This leaves the bulk of tissue potassium in a cellular potassium space which contains no sodium but whose potassium concentration is 113 to 138 mEq. per 1 kg. of tissue. We have no measure of true intracellular water concentration, but it is probably not far from the water concentration of the whole mucosa: 776 ml. per kilogram of tissue. This gives an intracellular potassium concentration of 146 to 178 mEq. per kilogram of H_2O, which is a reasonable range. When fragments of gastric mucosa are incubated in solutions containing various concentrations of potassium, the potassium concentration in the tissue behaves as if the cells were freely permeable to the ion, and in this respect they are similar to skeletal muscle cells. The mucosal surface of the cells may be less permeable than the serosal surface, for when K^{42} is present in the lumen of the dog stomach it leaves at the rate of only 0.11 per cent per minute.[1]

Water crosses the mucosa in both directions. When D_2O is placed on the mucosal surface of the nonsecreting stomach it exchanges for H_2O at the rate of 1.65 per cent per minute.[1] Approximately half the water in a nonsecreting stomach is insorbed in 30 minutes. However, sodium and hydrogen ions do not readily cross the mucosa along with the water. Radioactive sodium is insorbed from neutral solutions at the rate of only 0.47 per cent per minute, and it is insorbed from acid solutions at a slower rate.[1] Hydrogen ions diffuse through the mucosa in exchange for sodium ions, and HCl present in the lumen is very gradually converted to NaCl.[3] Organic anions such as lactate move asymmetrically across the mucosa, the more rapid flux being from mucosal surface to serosal,[15] and they may be exchanged for chloride ions actively transferred from blood to lumen by the chloride pump of the mucosa.[16]

Hydrogen ions move rapidly through the damaged barrier, and their flow is facilitated by exchange for sodium (fig. 2). Entry of hydrogen ions into the mucosa exacerbates damage caused by other agents, and sequential destruction of the mucosa follows. Potassium and organic cell contents enter the lumen, followed by sodium and chloride from interstitial fluid. Upon further destruction of barriers a fluid containing plasma proteins flows from interstitial spaces and capillaries. If plasma proteins can move through the mucosa in one direction, pepsin ought to be able to move through it in the other. Finally, after the most severe damage, bleeding occurs.

This description leaves many things

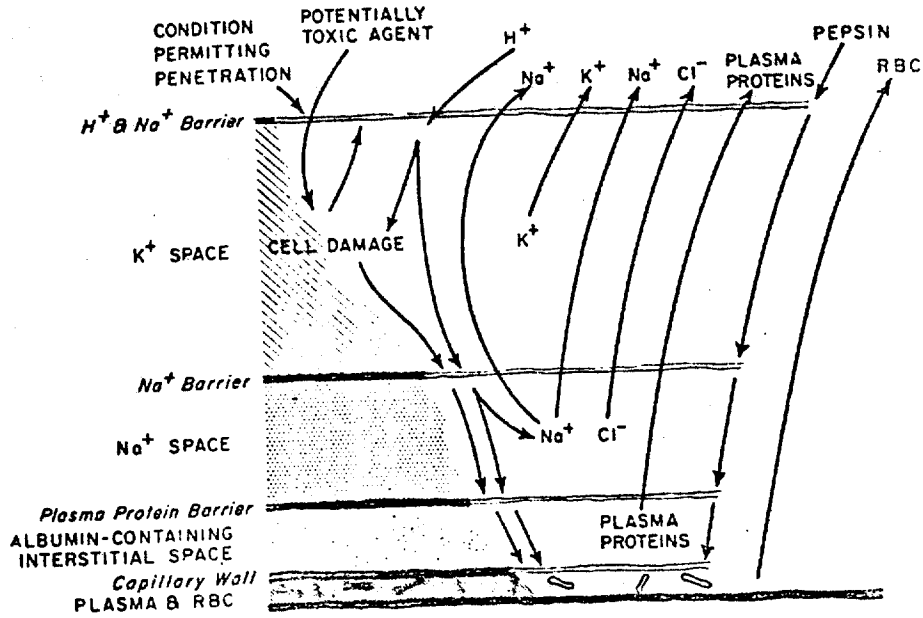

FIG. 2. Scheme of the sequence of events occurring during chemical injury to the gastric mucosa.

unexplained. We know very little about the intimate nature of the barriers, their loci, their physical and chemical structure, and their metabolic support. "Injury" itself is a vague term which covers events ranging from the most delicate molecular rearrangements to the most gross destruction of tissue. Although injury which allows extensive exchange of hydrogen and sodium ions must be diffuse, the points from which bleeding occurs are discrete. There must be local differences in susceptibility to injury, and there are certainly differences from animal to animal. We know nothing of the chemical events underlying repair and recovery. These problems can all be attacked experimentally, and we hope that by presenting this superficial description of chemical injury additional study will be provoked.

Summary

In 4 healthy, unanesthetized dogs, pouches of the oxyntic gland area of the stomach were irrigated for a 30-minute period with a solution of 100 mN of HCl and 15 mN of NaCl. Fluxes of H^+, Na, K, Cl, and H_2O were measured. The pouches were then irrigated for 2 30-minute periods with 100 mN acetic (or propionic or butyric) acid and 15 mN of NaCl. The organic acids rapidly moved through the mucosa, and fluxes of Na, K, and Cl from the mucosa were increased. These latter changes, plus increased flux of H^+ from pouch fluid, persisted in 2 subsequent 30-minute periods in which 100 mN of HCl was again used for irrigation, and they are interpreted as being signs of injury to the mucosa. No such changes occurred in control experiments in which neutral solutions containing the TRIS salts of the organic acids were used.

In similar experiments irrigation with 20 mN of acetylsalicylic acid in 100 mN of HCl caused bleeding and large changes in fluxes of H^+, Na, K, and Cl, but irrigation with neutral 20 mN of TRIS acetylsalicylate in 250 mN of glycine had no damaging effect.

It is concluded that the short chain fatty acids and acetylsalicylic acid damage the mucosa after they have diffused through a barrier and that to move through the barrier they must be in fat soluble form. A description of the sequence of events occurring in chemical injury is presented.

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tivity of NCP; (b) A 100% increase in total counts per minute recovered in collagen and a 50-60% increase in those of NCP per incubation; (c) a 700% increase in counts per minute in collagen and 200-300% increase in NCP per uterus; (d) A much higher ratio of specific activity of hydroxlysine from the treated rat to that from the untreated rat as compared with the ratio of specific activities of lysine from the same 2 groups of animals; (e) An effective dose of estradiol for stimulation of collagen synthesis is 1 μg with a dose-response time of 16 hours.

4. It is suggested that one of the important, if not the most important, action of estrogen in uterine connective tissue is concerned with the hydroxylation of lysine.

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Absorption of Short Chain Fatty Acids in Man.* (29505)

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Recently Barry and Smyth(1) investigated the absorption of short chain fatty acids from the gut of the rat and presented evidence to suggest that this was accelerated by an active transport system. Another explanation seemed possible(2) for diffusion of a compound across the gut wall besides being controlled by the compound's size and charge, is also modified by its lipid solubility(3,4).

We have, therefore, studied the relative rates of absorption of 7 short chain fatty acids from the human intestine and compared these with their partition into chloroform from an aqueous solution.

Patients studied and methods. The technique was similar to that used for studying sugar(5) and cortisol(6) absorption in man. The evening before the study the patients swallowed a double lumen polyvinyl tube ('Portex' M.L.T./B. external diameter 4.2 mm) to which had been attached a mercury bag. Next morning its position was checked fluoroscopically and the tube withdrawn slightly if necessary so that the mercury bag was approximately 40 cm past the duodenojejunal flexure. Fasting jejunal contents were collected from 7 patients. A solution contain-

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ing the mixture of acids was then pumped at 20 ml/min (using a Bowman constant infusion pump—Process and Instruments, Brooklyn, N. Y.) through the lumen which has an opening 35 cm from the end, *i.e.* the fluid entered the jejunum 5 cm past the duodeno-jejunal flexure. After 30 minutes equilibration samples of jejunal contents were siphoned off through holes cut into the other lumen of the tube 5 cm from the end, *i.e.* 30 cm below the point at which the solution entered the jejunum. The solution of acids also contained polyethylene glycol (PEG) molecular weight 4000 at a concentration of 0.5 g/100 ml. This substance is neither absorbed nor metabolized by the gut and may be used as a reference compound indicating dilution or concentration of the sample as it passes through the bowel(7): thus by knowing the concentration of acid in the aspirated and infused sample net absorption of acids/min/30 cm gut may be calculated.

The absorption of volatile acids from the colon was studied in a 55 year old woman who had had a colonic exclusion operation for chronic portal systemic encephalopathy (8). The operation leaves the patient with a fistula between the abdominal wall and the excluded colon. The colon was washed out by instilling 500 ml isotonic saline on 3 occasions and emptying after 10 minutes: on 2 further occasions 100 ml of a solution of short chain fatty acids and PEG were instilled and recovered after 10 minutes.

The fluid perfusing the gut contained the following short chain fatty acids, acetic, propionic, butyric, iso-butyric, valeric, iso-valeric and caproic in equimolar amounts, the final concentration being between 110 and 145 mM, and was made isotonic with plasma by addition of appropriate amounts of sodium chloride after the pH was adjusted to 7.0 with sodium hydroxide. Only a mixture of acetic, propionic, butyric, valeric and caproic acids were instilled into the colon: this solution was also isotonic at pH 7.0.

Analyses of samples. The pH of the aspirated sample was measured with a glass electrode. 1.5 ml $FeCl_3$ solution (1.0 g $FeCl_3 \cdot 6H_2O$ made up to 100 ml with water with addition of 2 drops of concentrated HCl) was added to 10 ml of intestinal sample, made up to 50 ml, shaken with 1.5 g $Ca(OH)_2$ and filtered. This precipitates protein and long chain fatty acids(9). Fifteen ml of the filtrate was acidified with sulphuric acid and the short chain fatty acids extracted with four 25 ml portions of ethyl-ether. The ether extract was dried with sodium sulphate and the acid content titrated with N/100 alcoholic KOH using bromthymol blue as the indicator.

The relative amounts of the individual acids were assessed by first adding enough dilute aqueous KOH to the ether extract to produce 2 phases—usually 3-4 ml. The aqueous extract of the sodium salts was then dried, and the liberated acids separated by gas chromatography(10). Polythylene glycol was estimated turbidometrically(11).

Partition of fatty acids into chloroform. On 3 occasions 10 ml of the equimolar solution of the short chain fatty acids (pH 7) such as was used in the perfusion experiments, was equilibrated against 400 ml chloroform. After standing overnight, the chloroform was run off into a flask, washed with 20 ml N NaOH and the alkaline aqueous solution of salts dried and analyzed in a similar way as were the intestinal samples.

Results. The mean fasting total short chain fatty acid concentration of the upper jejunal contents in the 7 patients was 7.7 mM (range 2-11.3) of which over 80% was acetic acid.

By the end of the perfusion there was some diarrhea on 4 occasions. This might have been due to direct irritation by the acids and their soaps, but in view of the fact that the solution was isotonic and its pH did not vary from 7 by more than 0.15, it is more likely that the solution acted as a bulk purge for under these conditions it was relatively poorly absorbed—only 0.89 (range 0.2-1.1) μMoles of short chain fatty acids were absorbed/min/30 cm jejunum.

In view of the fact that the small bowel had a measurable amount of acetate present in its lumen before the perfusion started, it was decided to express the rates of absorption in relation to propionic acid. Taking propionic acid as 1, it is seen from Table I

TABLE I. Relative Amounts of Short Chain Fatty Acids Recovered from the Jejunum and Colon, Compared with Their Partition into Chloroform.

	C_2	C_3	$IsoC_4$	nC_4	$IsoC_5$	nC_5	nC_6
Mean relative absorption							
Jejunum (7)*	1.6	1	.78	.72	.64	.54	.39
p†	.001	.001	.05	.02	.001	.001	
Colon (2)	1.41	1		.73		.59	.46
Partition into chloroform (3)		1	2.1	2.1	8.9	12.0	45

* No. of observations in parentheses. † Calculated by the paired t test.

that with increasing chain length there is relatively less of the acid recovered in the aspirated sample, thus the longer the fatty acid chain, the more quickly it was absorbed. The 2 isomers of butyric acid and valeric acid were also absorbed at different rates.

The 3 saline washings in the isolated colon contained 4 mM acetic acid before any acids were instilled, while after retention of the solution of the short chain fatty acids there was an overall absorption of 4 mMoles in 10 minutes, and the relative rates of absorption of the different acids were remarkably similar to those observed in the small bowel (Table I).

Partition of acids and salts into chloroform. The relative partition of the acids into chloroform from an equimolar solution at pH 7.0 is shown in Table I. With increasing chain length, there is increasing partition into chloroform.

Discussion. The relatively high concentration of acetate in the upper jejunal contents under fasting conditions may possibly be due to bacterial metabolism. This seems unlikely for the upper small bowel is said to be relatively sterile(12) and we have also found during absorption studies with sugars that after perfusing up to 6 l in 5 hours, the aspirated sample still contains comparable amounts of acetate. Under these conditions one would have expected bacteria and their products to have been swept down the gut. Another explanation is that the intestinal cells leak acetate into the bowel lumen.

The short chain fatty acids which we studied are weak electrolytes and their pK values are very similar, ranging from 4.76-4.85. This means that under the condition of perfusion at pH 7 the ratio of ionized/non-ionized acid is approximately 100/1. It is a general rule that ionized water soluble compounds do not diffuse across a membrane, but that their non-ionized components do(2,3,4). This is due to the greater lipid solubility of the non-ionized compound. Thus, relative absorption rate of weak electrolytes depend upon two factors, (1) their pK, which determines how much is ionized at a given pH, and (2) the relative lipid solubility of the non-ionized component. This latter has been tested for various drugs by equilibration against organic solvents such as heptane or chloroform (4). It must be realized that each solvent has slightly different properties which give only an overall indication of the properties of a lipid membrane surrounding cells. In our experiments, we equilibrated against chloroform at pH 7 to obviate even the slight difference in the pK of the acid. In fact the agreement between their partition into chloroform and their relative absorption rate is impressive, for although butyric and isobutyric acid partitioned equally into chloroform and yet had different rates of absorption, these were only significant at the 1 in 20 level. The very high ratio of acetate to propionate can probably be accounted for by the dilution of the perfusing fluid with the endogenous acetate.

The evidence cited by Barry and Smyth (1) that the rat small gut contains an active transport system for short chain fatty acids was: (1) *in vitro* preparations of rat small gut created a concentration gradient of the acids from the mucosal to serosal grade, and (2) the acids were absorbed at different rates. If in fact the main factor of importance in controlling the absorption of these acids were the relative lipid solubility of their

non-ionized form, then if *in vitro* the pH of the solution bathing the mucosa were lower than that bathing the serosa, a concentration gradient of the acids would be observed, for the serosal acids would be more ionized(2). Although Barry and Smyth(1) did not test this hypothesis, such a pH gradient has in fact been observed(13).

The fact that the absorption of these compounds from the large bowel followed the same pattern as the small bowel and that both could be predicted from their physical properties further suggests that it is not necessary to invoke an active transport system in man especially as the ability of the small bowel to absorb these acids is low, *e.g.* compared with glucose which, in spite of a higher molecular weight, is absorbed over 4 times as quickly. But it is possible that at such high concentrations as we used some inhibition of a transport system occurs(1).

Normally, these short chain acids are not an important feature of man's diet, although in ruminants they are a major product of cellulose fermentation in the rumen from where they are absorbed, metabolized and supply an important source of the animal's energy. It would seem that the greater the chain length the quicker they are absorbed by the squamous epithelium of the rumen (14).

In man, the main site of short chain acid formation is the large bowel, presumably the caecum, where they are most likely formed from bacterial action on cellular debris and unabsorbed carbohydrate. We have confirmed that faeces normally contain 10-20 mMoles/100 g wet weight(9,15) of which half is acetate, the rest being a mixture of the other short chain fatty acids and their isomers(9). Although this pattern may in fact reflect the metabolic pathways of colonic bacteria it may also reflect the more rapid absorption by the colon of the longer chain acids.

Summary. The relative rates of absorption of 7 short chain fatty acids from jejunum and colon of man have been compared with their partition into chloroform from an aqueous solution. The longer the chain length the faster the acids were absorbed. This probably reflected their greater solubility in lipid as shown by their partition into chloroform.

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The role of propionic acid as a precursor of methylmalonic acid in normal and vitamin B_{12}-deficient man

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In order to clarify the role of propionic acid as a precursor of methylmalonic acid (MAA) in the intact human, measurements of the incorporation of intravenously administrated tracer amounts of 2 ^{14}C-propionate into urinary ^{14}C-MMA, miscible MMA pool size, turnover, and urinary excretion were performed in vitamin B_{12}-deficient patients and suitable controls. While miscible MMA pool size is markedly increased in vitamin B_{12} deficiency (10 to 20 times), the fractional turnover rate is similar in controls and vitamin B_{12} deficiency. The daily absolute turnover and urinary excretion is proportional to MMA pool size. MMA specific activity following intravenous ^{14}C-propionate, however, was similar in all groups despite marked differences in pool size. These findings demonstrate that propionate is a precursor to MMA in normal and B_{12}-deficient man and, furthermore, suggest that the fraction of propionate going to methylmalonyl CoA and the fractional participation of propionate in relation to other precursors of methylmalonyl CoA is unchanged in vitamin B_{12} deficiency.

The increased methylmalonic acid (MMA) excretion of vitamin B_{12} deficiency[1-5] and certain congenital disorders[6-8] is the result of diminished isomerization of methylmalonyl-CoA to succinyl CoA by the vitamin B_{12}-dependent methylmalonyl-CoA isomerase system.[9, 10]

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Although propionic acid is a major precursor of methylmalonyl-CoA in ruminants,[11] its exact relationship to MMA in the intact man remains obscure.

The present study includes measurements of the incorporation of intravenously administered tracer amounts of 2-^{14}C-propionate into urinary ^{14}C-MMA, MMA pool size, turnover, and urinary excretion so as to determine the role of propionic as a precursor of MMA in man.

Methods and materials

Subjects consisted of a control group and a vitamin B_{12}-deficient group. The control group included 2 normal subjects and 2 patients with partially treated folic acid deficiency. The vitamin B_{12}-deficient group consisted of 4 patients with pernicious anemia. Also studied were 2 vitamin B_{12}-deficient patients 25 days and 10 months after the onset of vitamin B_{12} therapy. All subjects with vitamin B_{12}-deficiency had characteristic megaloblastic bone marrow changes. The vitamin B_{12} and folic acid status of the subjects was determined by serum vitamin B_{12}[12] and folate levels,[13] ^{57}Co-vitamin B_{12} absorption studies,[14, 15] daily urinary MMA excretion,[16] $^{14}CO_2$ breath excretion patterns after i. v. administration of 2(ring) ^{14}C-histidine and 2-^{14}C propionate[17, 18] and when possible, reticulocyte response to physiological therapy.[19, 20]

Fasting subjects received an intravenous injection of 10 to 15 μCi of 2-^{14}C-propionate (specific activity 8.15 mCi/mM.—radiochemical purity > 99 per cent)* and/or 25 μCi of 2-^{14}C-MMA (specific activity 1.9 mCi/mM., radiochemical purity > 98 per cent).† One vitamin B_{12}-deficient patient also received a 5.0 Gm. oral dose of sodium propionate 30 minutes prior to i. v. injection of 2-^{14}C-propionate. When multiple studies were performed on a single subject, a minimum of one week was allowed to elapse between studies. Fractional urine collections were obtained in bottles containing thymol for 48 hours after injection and were frozen at 20° C. until time of analysis.

Urinary MMA and ^{14}C-MMA specific activity were quantitated by methods previously described.[16] These methods include ether extraction, anion exchange chromatography, gel filtration, and spectrophotometric quantitation of the diazotized isolated MMA. ^{14}C-MMA specific activity was determined by further steps including thin-layer silica gel chromatography and liquid scintillation counting.

Urinary ^{14}C-MMA specific activity was expressed as a percentage of the radioactivity administered as either ^{14}C-propionate or ^{14}C-MMA per milligram of urinary MMA. MMA pool size and daily turnover was determined by in vivo isotope dilution techniques similar to that applied to the measurement of uric acid pool and turnover.[21] This technique involves serial measurement of urinary ^{14}C-MMA specific activity after intravenous injection of ^{14}C-MMA. The miscible MMA pool size is determined by relating the zero time extrapolated specific activity to the amount of ^{14}C-MMA injected. Turnover rate of the miscible MMA pool is determined from the slope of the serial ^{14}C-MMA specific activity determinations. The daily turnover of MMA is the product of the miscible MMA pool and its turnover rate.

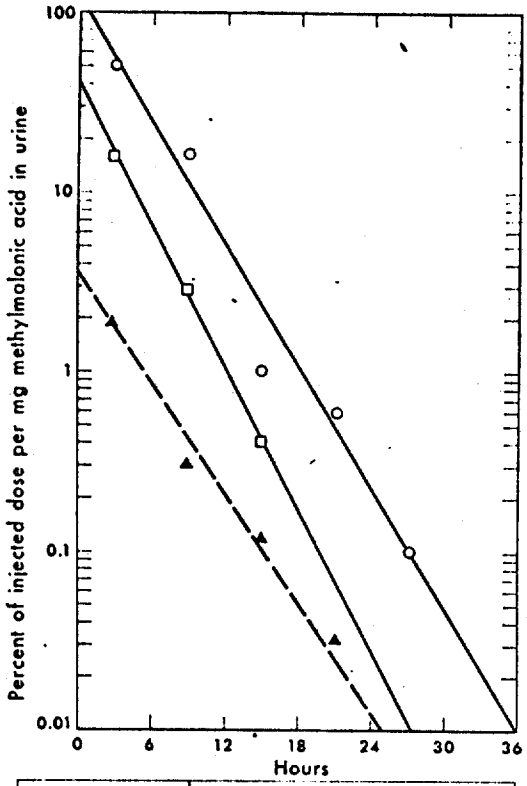
Results

While our primary objective was to determine the role of propionate as a precursor of MMA by quantitating ^{14}C-propionate incorporation into urinary MMA, knowledge of the MMA pool size and turnover aids in the interpretation of such data. Serial ^{14}C-MMA specific activity determinations on 6 hour fractional urine collections after i. v. injection of ^{14}C-MMA into 2 normal subjects and one patient with vitamin B_{12}-deficient megaloblastic anemia is shown in Fig. 1. The specific activity, expressed as a percentage of the injected dose of ^{14}C per

*Nuclear-Chicago Corp., Des Plaines, Ill.
†New England Nuclear Corporation, Boston, Mass.

milligram of MMA, is plotted against the mean time of each urine collection interval. During the 48 hour period of measurements, the ^{14}C-MMA specific activity is noted to decrease at a single exponential rate with half-times of 2.7 and 2.3 hours in the normal subjects and 3.0 hours in the vitamin B_{12}-deficient patient. The calculated miscible MMA pool, its fractional turnover rate, and quantitative daily turnover as well as the 24 hour urinary MMA excretion of these same subjects are also listed in Fig. 1. Miscible MMA pool size (27.8 mg.) in this vitamin B_{12}-deficient patient is approximately 10 to 20 times greater than that of the normal subjects (1.0 and 2.4 mg.).

It is of interest to note that the fractional turnover rate is similar in both normal controls and vitamin B_{12} deficiency, and consequently the daily turnover



	Pool size (mg)	Turnover rate (fract/day)	Turnover (mg/day)	Urinary excretion (mg/day)
○ CONTROLS	1.0	6.1	6.1	3.5
□	2.4	7.4	17.7	3.2
▲ B_{12} DEFICIENT	27.8	5.5	152.6	35.9

Fig. 1. Serial urinary ^{14}C-MMA specific activity measurements after i. v. injection of ^{14}C-MMA into one vitamin B_{12}-deficient patient, ▲; and two normal controls, ○ and □. The calculated MMA miscible pool size, turnover rate, absolute turnover, and MMA urinary excretion are listed for these subjects.

Table I. Urinary ^{14}C-methylmalonic acid after i. v. 2^{14}C-propionate

	Urinary methylmalonic acid (mg./48 hr.)	*Per cent injected dose in methylmalonic acid per 48 hr. (%)*	*Methylmalonic acid specific activity (% inj. dose/mg.)*
Normal controls (4)	10.3 (4.6-13.4)	0.05 (0.02-0.11)	6.5 × 10-3 (1.5-11 × 10-3)
Treated vit. B_{12} deficiency (1)	5.8	0.04	7.2 × 10-3
Partially treated vit. B_{12} deficiency (1)	73.7	0.55	7.5 × 10-3
Vit. B_{12} deficiency (4)	1,086 (54-3477)	2.51 (0.47-8.10)	6.4 × 10-3 (1.0-14.0 × 10-3)

Table II. Urinary ^{14}C-methylmalonic acid after i. v. 2^{14}C-propionate before an after propionate loading in a patient with vitamin B_{12}-deficient megaloblast anemia

	Urinary methylmalonic acid (mg./48 hr.)	*Per cent injected dose in methylmalonic acid per 48 hr. (%)*	*Methylmalonic acid specific activity (% inj dose/mg.)*
Normal diet	3,477	8.1	2.30 × 10-3
Normal diet plus 5 Gm. of sodium propionate	5,809	17.4	2.84 × 10-3

is proportional to the MMA pool size. Urinary excretion of MMA is also propo tional to pool size but represents only 20 to 50 per cent of the total turnove

Urinary MMA excretion and ^{14}C-MMA determinations after injection c trace amounts of 2^{14}C-propionate into 4 controls, fully treated and partiall treated patients with vitamin B_{12} deficiency, and 4 patients with vitamin B_{1} deficient megaloblastic anemia are shown in Table I. The fully treated patie had received 1000 μg of vitamin B_{12} i.m. monthly for 10 months. The partiall treated patient received 1 μg of vitamin B_{12} i.m. per day for 25 days. The resul are expressed in terms of 48 hour urine collections after administration of t ^{14}C-propionate, because it was found that essentially all of the excretion c radioactivity in the MMA fraction occurred within 48 hours. In all the subjec studied, incorporation of the 2-carbon atom of propionate into urinary MMA noted to occur. While there is a marked increase in MMA pool size and turnove in the partially treated and vitamin-deficient patients, no significant differenc in ^{14}C-MMA specific activity was noted between these patients and the control Therefore, the increased percentage excretion of the injected dose as ^{14}C-MM in the partially treated and vitamin B_{12}-deficient patients was due to the i creased excretion of MMA in these patients.

The effects of a 5.0 Gm. oral propionate load on urinary MMA excretion and ^{14}C-MMA determinations after injection of ^{14}C propionate in a vitamin B_{12}-deficient subject are shown in Table II. On a normal diet this subject excreted very large amounts of MMA. While the oral propionate load resulted in a marked increase in MMA excretion and MMA pool size, no significant change is noted in the ^{14}C-MMA specific activity. Thus doubling of the MMA pool size by propionate loading in the same subject did not change ^{14}C-MMA specific activity. This constancy of specific activity in a single subject is similar to that noted in the subjects with widely varying MMA pool size and urinary excretion (Table I).

Discussion

The variable degree of excessive methylmalonic aciduria noted in vitamin B_{12} deficiency suggests that there may be multiple dietary and endogenous precursors of urinary MMA in man. Thus the excessive degree of methylmalonic aciduria noted in a given patient may be not only dependent upon the degree of metabolic block but also upon the types and amounts of available precursors which may vary from patient to patient. Substances that have been considered as possible precursors of MMA in man include propionic acid and other odd-numbered fatty acids, isoleucine, valine, cholesterol, and possibly pyrimidine catabolites.[22-24]

Propionic acid, a product of rumen bacterial action via its activation to propionyl-CoA, is a major precursor of methylmalonyl CoA and a major source of energy in ruminants.[11] The quantitative relationship of propionic acid to MMA in man is unknown. While the oral intake of valine has a relatively predictable effect on urinary MMA output in patients with diminished methylmalonyl-CoA isomerase activity,[23] oral loading doses of sodium propionate have failed to uniformly and predictably increase urinary MMA excretion.[2, 23, 25] Although the oxidation of 2-^{14}C-propionate has been found to be abnormal in human vitamin B_{12} deficiency, the degree of oxidative abnormality did not correlate with the degree of methylmalonic aciduria. Furthermore, after adequate vitamin B_{12} therapy, the oxidative abnormality persisted long after the urinary MMA excretion returned to normal.[18]

The results of the present tracer studies (Tables I and II) and loading study indicate that propionate is a precursor to urinary MMA and therefore intracellular methylmalonyl-CoA in both normal and vitamin B_{12}-deficient human subjects. While the percentage of injected activity found in urinary MMA was relatively small, especially in the cases of the nonvitamin B_{12} subjects, the MMA pool size and turnover studies indicate that urinary excretion accounts for only approximately 20 to 50 per cent of the daily MMA turnover in normal subjects and approximately 20 per cent in vitamin B_{12} deficiency (Fig. 1). The metabolic fate of the remainder of the daily MMA turnover is unknown. Preliminary studies in this laboratory have shown that after injection of ^{14}C-MMA, 20 per cent of the dose is expired in the breath as $^{14}CO_2$.

The marked augmentation of the methylmalonic aciduria by oral propionate loading in the vitamin B_{12}-deficient patient in the present study is noteworthy as prior attempts to increase the methylmalonic aciduria in vitamin B_{12} deficiency

by such loading have resulted in inconstant and minimal increases in urinary MMA.[2, 23, 25] The fact that this patient had an extreme degree of methylmalonic aciduria prior to loading may in some way relate to the marked augmentation after loading. Diminished catabolism of propionate by normally operative metabolic pathways not dependent upon vitamin B_{12} could explain this occurrence.[22, 26]

Even though there is a marked increase in the MMA pool in vitamin B_{12} deficiency, there was no significant difference in urinary ^{14}C-MMA specific activity after injection of ^{14}C-propionate into controls and vitamin B_{12}-deficient subjects, or change in ^{14}C-MMA specific activity after increase in pool size by oral propionate loading. These findings indicate that the fraction of propionate pool going to methylmalonyl-CoA and the fractional participation of propionate in relation to other precursors of methylmalonyl-CoA is unchanged in vitamin B_{12} deficiency.

These results suggest that the methylmalonyl-CoA pool size may not be appreciably altered by vitamin B_{12} deficiency even though there is diminished isomerization of methylmalonyl-CoA to succinyl CoA. A compensating increase in the rate of deacylation of methylmalonyl-CoA to MMA would maintain the methylmalonyl-CoA pool at its normal size.

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used was prepared in this buffer. It was found that the compound is a powerful inhibitor of nitrite oxidation and the inhibition produced by different concentrations is shown in Table 1.

Table 1. INHIBITION OF NITRITE OXIDATION BY 2,4-DINITROPHENOL

Concentration of 2,4-dinitrophenol (m*M*)	Percentage inhibition of oxygen uptake
0·3	16
0·6	23
1·2	64
1·8	80
2·4	94

One possible explanation of the effect of 2,4-dinitrophenol is that, by virtue of its nitro groups, it can compete with nitrite for the nitrite-oxidizing enzyme. However, it was found that variation of the initial nitrite concentration between 6 and 44 m*M* had little effect on the degree of inhibition produced by a given concentration of 2,4-dinitrophenol. It therefore seems unlikely that such a competition between nitrite and 2,4-dinitrophenol occurs.

Another possibility is that 2,4-dinitrophenol inhibits because of its well-known ability to uncouple oxidative phosphorylation[3]. Although it has yet to be proved, it is almost certain that nitrite oxidation by *Nitrobacter* is linked to the production of high-energy phosphate bonds. If this phosphorylation is coupled in an obligatory way, then any uncoupling agent should inhibit nitrite oxidation. The effect on nitrite oxidation of two dyes, janus green and brilliant cresyl blue, both of which have been shown to uncouple oxidative phosphorylation in mammalian systems[4], was therefore tested, but neither had any effect at concentrations up to 10 m*M*. It is possible, however, that neither dye could penetrate to the site of nitrite oxidation within the cell.

The effect on nitrite oxidation of a few compounds related to 2,4-dinitrophenol was also tested, and the results are given in Table 2. All conditions were the same as in the experiments with 2,4-dinitrophenol; each compound was used at a concentration of 1 m*M*.

Table 2. EFFECT ON NITRITE OXIDATION OF SOME COMPOUNDS RELATED TO 2,4-DINITROPHENOL

Compound tested	Percentage inhibition of oxygen uptake
2,5-Dinitrophenol	61
m-Nitrophenol	77
p-Nitrophenol	100
p-Nitroaniline	25
Phenol	7

It is of interest that the nitrophenols produced the greatest inhibition, and it appears that both the phenolic hydroxyl and nitro groups are necessary to give the maximum effect. As in the case of 2,4-dinitrophenol, inhibition may be due to a direct action on the nitrite oxidizing enzyme, or to an uncoupling of oxidative phosphorylation.

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PHYSIOLOGY

Inhibition of Appetite in Dairy Cattle by Certain Intermediate Metabolites

MANNING *et al.*[1] have discussed the applicability of the glucostatic theory of Mayer *et al.*[2] to the regulation of food intake in sheep. Glucose in ruminant animals plays a relatively minor part in energy metabolism, since a major portion of ingested carbohydrates are degraded to volatile fatty acids which are absorbed into the blood stream via the rumen wall. Consequently, a chemoreceptor mechanism of regulation of intake in ruminants may depend on any of several metabolites. The objective of this experiment was to examine several major intermediate metabolites for their effect on appetite.

Sodium acetate (adjusted with acetic acid to pH 7), glucose, acetic, propionic, valeric, hexanoic and lactic acids were administered intravenously by constant drip over the same 8-hr. period during each of three consecutive days. In each trial, identical twin cows of the Guernsey, Holstein-Friesian or Jersey breed were injected. One twin received the metabolite in three litres of physiological saline and the other received an equal amount of physiological saline. The injections were made into the jugular vein through a small polyethylene catheter inserted previously that remained in place throughout each trial. The catheter was firmly attached to the shoulder of the animal, thus allowing all manipulations to be made at that position. This technique permitted the elimination of any restraining apparatus other than a halter, to which the animals were already accustomed. The injection procedure and experimental manipulation apparently did not affect intake, since the control twins consumed the same amount of feed during the three-day injection period as they did during the three days prior to injection. The effect on appetite was determined by comparing the voluntary hay intake of chopped alfalfa of the control twin (saline injected) with that of the treatment twin (metabolite injected). The relative amounts of each metabolite administered are shown in Table 1. These amounts are expressed as a percentage of body maintenance requirement[4]. So far as possible, the higher levels of organic acids used were those corresponding to what would seem to be equal to, or in excess of, the actual amounts absorbed from the gastro-intestinal tract during an 8-hr. period.

Administration of glucose did not elicit a reduction in daily intake. This is in agreement with the observation of Vallance and McClymont[5] during injection of 4 kgm. of glucose over a 48-hr. period, and that of Manning *et al.*[1] following intravenous administration of 50 per cent glucose to sheep. In the case of the higher (25 per cent) level, concentrations of blood glucose were raised from approximately 40 mgm. per 100 ml. to approximately 100 mgm. per 100 ml., or about 2½ times normal. From these results, it is clear that raised levels of blood glucose have little or no effect on the appetite of dairy cattle.

The administration of the higher level of acetic acid and sodium acetate resulted in a highly significant reduction in feed intake during the 8-hr. injection period; with both metabolites consumption was negligible. Consumption during the other 16 hr. of the three injection days approached normal. However, the 24-hr. feed-intake values during the three days were significantly lower for the treatment

Table 1. EFFECT OF INTRAVENOUSLY ADMINISTERED METABOLITES ON CONSUMPTION OF FEED BY IDENTICAL TWIN COWS

Metabolite	Level*	No. of trials	Average intake during 8-hr. injection period, 3 days. Treated twin (lb.)	Average intake during 8-hr. injection period, 3 days. Control twin (lb.)	Average 24-hr. intake including 8-hr. injection period, 3 days. Treated twin (lb.)	Average 24-hr. intake including 8-hr. injection period, 3 days. Control twin (lb.)
Glucose	4·0	2	8·2	9·2	19·5	18·8
Glucose	25·0	2	5·8	6·4	16·4	15·8
Sodium acetate	12·5	2	0·5§	8·4	14·0†	20·2
Acetic acid	12·5	1	0·8§	6·3	3·1‡	17·9
Lactic acid	4·0	1	14·8	14·1	27·1	28·6
Acetic acid	3·5	1	6·8	9·3	18·1	16·6
Propionic acid	12·5	2	2·1§	10·0	15·1‡	23·6
Butyric acid	6·25	2	14·7	14·7	27·6	28·0
Valeric acid	6·25	2	15·4	18·8	30·4	32·8
Hexanoic acid	6·25	2	12·0	12·6	28·8	29·6
Lactic acid	6·25	2	19·8	20·4	32·2	34·6

* Percentage of body digestible energy maintenance requirement. † Significant ($P < 0·05$). ‡ Significant ($P < 0·01$). § Significant ($P < 0·005$).

twins than for the control twins. Administration of propionic acid resulted in significant reductions in intake during the 8-hr. and 24-hr. periods. There was very little carry-over effect from acetate, acetic or propionic acid injections, since eating began very soon after cessation of each 8-hr. injection period.

None of the other metabolites mentioned above caused a significant reduction in feed intake. Valeric acid and lactic acid injection resulted in a small mean reduction in intake, but the results were variable and exhibited no definite trend.

The results of this series of trials strongly suggest the existence of a chemoreceptor response to changes in blood constituents. Apparently, one of the possible mechanisms of regulation of intake involves the absorption of acetic acid and propionic acid or, as indicated by the experiment using sodium acetate, the acetate and propionate ions, into the blood stream which, at threshold-levels, would elicit a chemoreceptor response inhibiting intake. Acetic and propionic acid inhibition of feed intake may account for the observation that dairy cattle consume less forage in the form of silage than in the form of hay, since silage contains significant amounts of these acids.

The level of feed consumption (highly variable in ruminants) is of the utmost importance, since gross efficiency of milk production is limited by voluntary intake of nutrients. From the results of this experiment, it is postulated that rations tending to promote the production of metabolites serving as energy sources that do not inhibit nutrient consumption could greatly enhance the gross efficiency of animal production by promoting an increase in voluntary intake of nutrients.

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Electrical Inexcitability of the Synaptic Membrane in the Frog Skeletal Muscle Fibre

VARIOUS types of evidence indicate that the endplate potential of frog twitch muscle fibres is an electrically inexcitable synaptic activity[1,2]. Electrical inexcitability of the synaptic membrane has been demonstrated most clearly in cells in which electrically excitable activity is lacking, for example, the electroplaques of marine electric fishes[3] and slow muscle fibres of frog[4]. Electrical inexcitability of synaptic membranes in other cells has also been demonstrated[1,2]. Hitherto, however, it has not been shown that the chemically excitable[5] synaptic membrane of twitch muscle fibres is also unresponsive to electrical stimuli. The junctional region is small, and there is intermingling of membrane of greater and lesser chemical sensitivity[5].

Experiments described here demonstrate that sites of the junctional membrane do not develop an inward current when a directly evoked spike is propagated through the endplate region. These are also the sites with the largest and most rapidly rising externally recorded spontaneous miniature endplate potentials.

Most of the work was done on whole sartorius muscles of frog (*Rana pipiens*). A few experiments were performed on isolated single nerve–muscle fibre preparations from the semitendinosus muscle, the results being identical in both series. The preparations were bathed in hypertonic saline, made by doubling the sodium chloride of a standard frog Ringer's solution. Hypertonicity served two functions: decreasing the contraction of the muscle[6] and increasing the frequency of spontaneous miniature endplate potentials[7]. The latter effect aided location of synaptic sites, which appear to be distributed as small patches in the junctional region[5].

Superficial nerve endings to a neuromuscular junction were visualized, and a recording micro-electrode was inserted into this region of the muscle fibre. Spikes were elicited by depolarizing pulses which were applied through another intracellular electrode in the same muscle fibre at some distance away from the junction. An external recording micro-electrode was used to explore the endplate region for discrete areas of spontaneous sharply rising external negativities. The potentials were recorded with direct coupled amplifiers.

When the external micro-electrode was localized at a site which yielded rapidly rising outside-negative miniature endplate potentials, the potential recorded during the spike was purely positive (Fig. 1*A*). On moving the micro-electrode in the long axis of the fibre less than 5μ from this site a brief dip developed in the positivity (Fig. 1*B*). The dip became larger when the electrode was moved slightly farther away (Fig. 1*C*). Negativity developed on moving the electrode another 30μ (Fig. 1*D*). Further displacement of the electrode by about 100μ resulted in a potential with a very large negativity (Fig. 1*E*). This last was the potential usually recorded outside the muscle fibre at non-synaptic regions.

Invasion of the junctional region by the directly evoked spike was confirmed by the records obtained with the intracellular micro-electrode (Fig. 1*F*). The externally probed site which gave the purely positive potential of Fig. 1*A* was shown to give rise to spontaneous negativities both before and after the spikes

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Effect of Acetate and Propionate on the Utilization of Energy by Growing-Fattening Lambs [1]

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ABSTRACT Growing-fattening lambs fed a basal ration of pelleted alfalfa hay showed no response in either rate of gain in body weight or feed required per unit gain when propionate or acetate was incorporated in the feed. The addition of corn to the basal ration resulted in a significantly faster rate of gain. Estimates of the composition of gains were obtained by slaughter and chemical analysis of 4 lambs at the beginning, and 2 lambs from each treatment group at the end of the trial. The gains of the lambs fed propionate, acetate or corn were considerably higher in fat than those of controls. On an energetic basis, these groups converted digestible energy to body gain with considerably greater efficiency than the control group. No difference in efficiency was observed among supplements.

Calorimetric studies of Armstrong and Blaxter (1) and Armstrong et al. (2) have indicated clearly that propionic and butyric acids are utilized with greater efficiency than acetic acid when infused into the rumen of fattening sheep. On the basis of this work it might be postulated that an increase in either rate of gain or feed efficiency, or both, would result from the addition of propionic or butyric acid to rations which normally yield high proportions of acetic acid during ruminal fermentation. In general, however, addition of the free acids to rations has rendered them unpalatable (3, 4) and the acids when added as salts have not resulted in significant differences in rates of body gain in lambs (3, 5, 6). In beef cattle the addition of 2% sodium propionate to fattening rations did not improve gains over controls, although it tended to reduce feed intake and increase feed efficiency slightly (7). Intra-ruminal infusions of acetic, propionic and butyric acids in non-lactating dairy heifers fed a normal ration resulted in increased nitrogen retention and body weight gain, but in no case was the difference among acids significant (8).

The possibility remained that dietary propionate or butyrate might result in a greater gain of body fat than acetate, without influencing rate of gain in weight. In the experiment reported here complete carcass analyses were made to compare the composition of gains in lambs fed a basal roughage ration with or without additions of acetate, propionate or corn.

EXPERIMENTAL PROCEDURE

Sixty-three native (New York State crossbred) shorn, feeder lambs (23 to 35 kg) were divided as to sex and initial weight and randomly assigned to 9 groups of 7 lambs each. The groups were then allotted at random to 4 dietary treatments (2 groups per treatment). Four lambs, representing the weight range, from the remaining group were killed for analysis at the beginning of the trial. The 3 remaining lambs in this group were discarded. Each group was assigned a separate pen and group-fed twice daily on an ad libitum basis for a 50-day feeding period. Feed consumption and refusals were recorded daily and body weights to the nearest 0.2 kg were taken weekly. Water and trace-mineralized salt were available throughout the trial. At the end of the trial, one representative lamb from each pen (2 per treatment) was selected, shorn, and killed for analysis.

The 4 experimental rations employed were: 1) alfalfa hay, 2) alfalfa hay plus 113 g shelled corn per lamb per day, 3) alfalfa hay containing 10% sodium propionate ($NaC_3H_5O_2$), and 4) alfalfa hay con-

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taining 10% sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$).

The feeds were prepared and fed in complete pelleted form, except for the shelled corn, which was added in appropriate quantity at each feeding. Alfalfa hay, the basal portion of all rations, was ground in a hammermill through a 0.3-cm screen. A portion of this was pelleted in a mobile pelleting unit through a 2-cm die. After repeated, unsuccessful efforts with the mobile machine to pellet the mixture of ground hay and sodium acetate or propionate, this material was pelleted in a commercial feed mill through a 0.6-cm die. As a result of these difficulties the basal pellets used in the trial were of a different diameter than those containing the propionate and acetate, although all were made from the same lot of ground hay. Following the growth trial, 4 of the lambs were used to assess the digestibility of the 4 rations.

Feed samples were composited during the growth trial and during each period of the digestion trials. These, together with the dried fecal samples were subjected to proximate analysis and heats of combustion were determined in a Parr adiabatic oxygen bomb calorimeter. The shorn, complete empty bodies of the lambs killed were frozen, ground, mixed and sampled in duplicate for analysis. These samples were freeze-dried to constant weight for moisture determination and subsequently extracted with diethyl ether. The dried extracted material was analyzed for moisture, ether extract, nitrogen and ash, and heats of combustion were determined as described above. Energy content of the empty bodies was computed by assigning a value of 9.405 kcal/g [3] to the extracted fat, and using the heats of combustion of the dried "fat-free" material. The initial composition and energy content of the ingesta-free, wool-free bodies of each group of lambs were estimated by computing the mean composition and caloric value per unit live weight of the 4 lambs initially slaughtered and applying these values to the mean initial weights of the groups. Final composition and energy content were estimated in the same manner. The composition and energy of the wool were not estimated, although wool weights were recorded.

RESULTS AND DISCUSSION

Proximate composition of the feeds used is presented in table 1 and body weight gains and feed consumption data in table 2. The ash values for the feeds containing acetate or propionate were higher than would be expected from the added sodium. They were, however, consistent among feed samples and may reflect the partial oxidation of sodium in the ash determination. The lambs receiving alfalfa hay and corn gained significantly ($P < 0.01$) more body weight than those on the other treatments. Average body weight gains of those on the basal, acetate and propionate treatments were almost identical. This observation is in substantial agreement with earlier work (5). An examination of the data on gastrointestinal "fill" of the lambs killed, indicated only small variation among treatments (range 21 to 24% of live weight). Wool weights likewise showed

[3] Paladines, O. L. 1963 Energy utilization by sheep as influenced by physical form, composition and level of intake of diet. Ph.D. Thesis, Cornell University.

TABLE 1

Proximate composition of feeds

Feed	Water	Crude protein	Ether extract	Crude fiber	Nitrogen-free extract	Ash	Organic matter
	%	%	%	%	%	%	%
Alfalfa pellets	12.1	14.0	1.8	32.5	34.1	5.6	82.4
Alfalfa + propionate [1]	15.4	12.8	1.7	28.8	30.7	10.6	74.0
Alfalfa + acetate [2]	16.4	12.8	1.7	29.6	30.6	8.9	74.7
Shelled corn	13.2	8.8	4.5	2.4	70.0	1.1	85.7

[1] Sodium propionate N.F. ($NaC_3H_5O_2$) added to ground hay at 10% level; propionic acid equivalent approximately 7.7% of the feed.
[2] Sodium acetate N.F. ($NaC_2H_3O_2 \cdot 3H_2O$) added to ground hay at 10% level; acetic acid equivalent approximately 4.4% of the feed.

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ogen-ree ract	Ash	Organic matter
%	%	%
1.1	5.6	82.4
0.7	10.6	74.0
0.6	8.9	74.7
0.0	1.1	85.7

el; propionic acid equiva-
el; acetic acid equivalent

little variation (range 540 to 650 g). Slightly less feed was consumed by the lambs on the acetate treatment, and particularly those receiving the propionate treatment, than by the control or corn groups. This may be attributable to the higher sodium content of these diets (4). The average calculated consumption of acetic and propionic acids in the rations was very nearly equal on a molecular basis; on a caloric basis, however, the lambs on the propionate treatment consumed in the form of organic acid almost twice as much energy as those on the acetate treatment.

The addition of acetate or propionate to the rations had little effect on the digestion coefficients for any of the proximate components (table 3), with the possible exception of crude fiber. Acetate appeared to reduce crude fiber digestibility slightly, but the reduction was not statistically significant. The total digestible nutrient (TDN) values for the feeds containing acetate and propionate were substantially lower than the TDN value of the basal feed primarily because of their higher ash content. On an ash-free basis the TDN values were 53.8, 53.4 and 50.9 for the basal, propionate and acetate treatments, respectively. The digestion coefficients for the shelled corn were obtained in "difference" trials, in which corn was fed at low levels with the basal pelleted ration. The coefficients for corn were extremely variable among animals as is frequently the case in such trials; however, the average values are in reasonably good agreement with those for No. 2 dent corn as listed by Morrison (9). The average TDN value is very close to Morrison's value on a dry-matter basis and was, therefore, considered valid for use in efficiency calculations.

The data in table 4 show that the lambs from the propionate, acetate or corn treatments were fatter than those from the basal ration. This is reflected in both the composition and energy content of the dry matter of their ingesta-free, wool-free bodies. Furthermore, there was essentially no difference in composition of those on the acetate and propionate treatments. While

TABLE 2

Average body weight gains and feed consumption

	Ration			
	Basal	Propionate	Acetate	Corn
No. of lambs	14	14	14	14
Initial weight, kg	27.7	28.0	28.1	27.6
Final weight, kg	35.7	35.4	35.6	37.3
Daily gain, kg [1]	0.16	0.15	0.15	0.19
Feed consumed, kg	87.6	79.4	84.3	86.8
TDN [2] consumed, kg	38.8	31.4	32.1	40.5
DE [3] consumed, megcal	191.6	181.9	177.7	197.6

[1] SE of mean = 0.012. Corn > others ($P < 0.01$).
[2] Total digestible nutrients.
[3] Digestible energy.

TABLE 3

Digestion coefficients, total digestible nutrient (TDN) and digestible energy (DE) values of the feeds

Feed	Dry matter	Crude protein	Ether extract	Crude fiber	Nitrogen-free extract	Energy	TDN [1]	DE
	%	%	%	%	%	%	%	*kcal/kg*
Alfalfa pellets	51.2 [2]	65.6	49.6	33.8	64.9	52.6	50.4	2487
Alfalfa + propionate	52.9	66.7	44.3	32.5	64.2	55.8	46.7	2709
Alfalfa + acetate	50.1	64.4	46.7	30.4	62.7	52.5	45.5	2521
SE [3]	±1.02	±1.40	±5.71	±1.38	±1.38	±0.99	±0.93	±25
Shelled corn [4]	89.1	73.8	85.0	50.4	93.7	87.7	92.8	4095

[1] Dry-matter basis.
[2] Each coefficient is the average of 3 observations.
[3] SD of a mean.
[4] Values determined by difference. The standard errors are not appropriate to the values for shelled corn.

those receiving corn gained more weight and thus had a higher total body energy content than those on the fatty acid treatments, they were not appreciably different in composition. Those fed the basal ration, however, were similar in composition to the lambs analyzed at the beginning of the trial. Bentley et al. (5) presented data on the moisture and fat content of the ground shoulders from lambs fed acetate or propionate which, though quite variable, suggested that those fed the acid salts were fatter than controls.

The estimated composition of the gains, as well as the estimated total energy gains for the entire groups are presented in table 5. These data suggest that the gain in body weight of the basal group was much higher in water and lower in fat than that of the other 3 groups, all of which were very similar. The validity of these data is obviously predicated on the assumption that the lambs analyzed were typical of the lambs in the groups they represented. While compositional data are, of course, unavailable to support this assumption, the average rate of body weight gain of the 2 lambs slaughtered in each case closely paralleled the average rate of gain of their respective treatment groups.

Calculations of efficiency of gain (table 6) indicate that the feed required per unit of gain in live weight was similar for the basal, acetate and propionate rations, and somewhat lower for the ration containing corn. When the caloric efficiency was computed on the basis of digestible energy (DE) required per unit energy gain, the basal ration was less efficiently utilized than the other three, all of which were similar. Digestible energy intakes were very similar with the acetate and propioate treatments. The fact that the lambs consumed nearly twice as many calories from propionate as they did from acetate plainly suggests that the propionate was not utilized with greater energetic efficiency than the acetate.

The lack of agreement between these results and those of Armstrong et al. (1, 2) is not readily explained. Several factors, however, may have relevance. The animals used by Armstrong et al. (1, 2) were adult sheep; those in the present study

TABLE 4

Average chemical analyses of ingesta-free, wool-free lamb bodies

Treatment	No. lambs	Dry matter	Composition, dry-matter basis			
			Crude protein [1]	Ether extract	Ash	Total energy
		%	%	%	%	*megcal*
Initial	4	38.0	42.4	48.1	11.9	54.6
SE [2]		±1.2	±2.0	±2.7	±0.7	±4.24
Final						
Basal	2	35.5	43.6	46.7	10.4	65.1
Basal+propionate	2	38.8	39.1	52.0	9.4	76.8
Basal+acetate	2	38.6	39.6	51.6	9.9	76.0
Basal+corn	2	38.4	38.5	53.0	9.4	80.5
SE		±1.7	±2.9	±3.8	±1.0	±5.99

[1] N × 6.25.
[2] SD of a mean.

TABLE 5

Estimated composition and total energy of ingesta-free, wool-free body gains

Treatment	Water	Fat	Protein	Ash	Total energy
	%	%	%	%	*megcal*
Basal	73.8	11.0	14.0	1.3	11.7
Basal+propionate	60.9	26.0	12.2	1.0	21.6
Basal+acetate	60.5	24.7	13.1	1.8	21.7
Basal+corn	61.2	25.3	12.0	1.5	27.1

ıtered in each case closely
: average rate of gain of their
atment groups.
ıs of efficiency of gain (table
ıat the feed required per unit
e weight was similar for the
: and propionate rations, and
ver for the ration containing
the caloric efficiency was
the basis of digestible energy
d per unit energy gain, the
was less efficiently utilized
er three, all of which were
estible energy intakes were
with the acetate and propio-
s. The fact that the lambs
arly twice as many calories
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with greater energetic effi-
ne acetate.
agreement between these re-
se of Armstrong et al. (1, 2)
explained. Several factors,
y have relevance. The ani-
Armstrong et al. (1, 2) were
th ...n the present study

ree lamb bodies

sition, dry-matter basis		
Ether extract	Ash	Total energy
%	%	*megcal*
48.1	11.9	54.6
±2.7	±0.7	±4.24
46.7	10.4	65.1
52.0	9.4	76.8
51.6	9.9	76.0
53.0	9.4	80.5
±3.8	±1.0	±5.99

. wool-free body gains

	Ash	Total energy
	%	*megcal*
	1.3	11.7
	1.0	21.6
	1.8	21.7
	1.5	27.1

TABLE 6

Efficiency of feed utilization

Criteria	Treatment			
	Basal	Basal + propionate	Basal + acetate	Basal + corn
Feed/kg gain, kg	11.0	10.6	11.4	8.9
TDN [1]/kg gain, kg	4.9	4.2	4.3	4.2
TDN/megcal gain, kg	3.32	1.45	1.48	1.49
DE [2]/megcal gain, megcal	16.38	8.42	8.19	7.29
Net utilization of DE for gain,[3] %	11.7	24.0	25.4	26.0

[1] Total digestible nutrients.
[2] Digestible energy.
[3] Retention of energy surplus to maintenance; maintenance requirement estimated as 76 $W^{0.75}$lb (Garrett et al. (11)). Energy gain in wool disregarded.

were growing-fattening lambs. Using growing heifers infused with fatty acids, Rook et al. (8) failed to obtain a significant difference between acetic and propionic acids in calculated energy gain. In the present study the acids were incorporated in the feeds as acid salts, whereas in the British work they were infused as dilute acids into the rumen. Although the acid salts did not appear to grossly alter digestibility, their possible effect on the rumen environment should not be overlooked as a factor contributing to the difference in results. It is interesting in this respect that Bentley et al. (5) reported a lower total amount of volatile fatty acids in the rumens of lambs fed propionate than of those fed acetate.

The net utilization of DE surplus to maintenance was computed (table 6) using the formula of Garrett et al. (11) to estimate maintenance requirements. When expressed in this way the efficiency of energy utilization is again very similar for the 3 supplemented groups. Approximately one-half of the DE consumed was required for maintenance. The evidence available (10) indicates that mixtures of volatile fatty acids of widely varying proportions are utilized with essentially equal efficiency for this purpose. It may be that the present study was not sufficiently sensitive to detect differences between the acetate and propionate rations at the levels employed.

The striking difference in efficiency between the basal and supplemented groups is also difficult to explain. The basal group consumed more DE than either the acetate or propionate groups. If it is assumed that the added acetate and propionate were quantitatively absorbed without the energy losses normally accompanying ruminal fermentation, they would account for approximately 7 and 13%, respectively, of the DE consumed. The elimination of the energy loss associated with fermentation of such a small portion of the caloric intake appears to be much too small to account for the marked change in energy gain. Bentley et al. (5), however, reported very high feed replacement values for acetate and propionate in their studies. The results of the present experiment indicate that acetate, propionate or corn when added to an all-hay ration greatly increase the efficiency with which energy is stored in the growing-fattening lamb.

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THE AMOUNTS OF SHORT CHAIN ACIDS FORMED DURING RUMEN FERMENTATION [1]

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RUMINANTS have long been known for their ability to utilize roughage. The advent of intensive animal production and increased concentrate feeding, having fostered anomalies in rumen fermentation. These include changes in the production of short chain acids which are a major source of energy for the cow as well as an end product of the fermentation. Information concerning amounts and types of these acids formed in the rumen is imperative to a better understanding of feed utilization. This study was undertaken to determine the quantity of short chain acids made available to the ruminant through fermentation of the roughage and concentrate in the ration. The turnover curve method used in this study is an application of the general method described by Solomon (1953).

Experimental

Twin Holstein cows, T1 and T2, weighing 1050 and 1110 lb. and a Guernsey steer, C707, were used in this experiment. The steer weighed 1420 lb. when used for the restricted roughage trial and 1500 lb. for the all hay trial. All three animals were fitted with fistula plugs described by Hentschl *et al.* (1954). T1 was in the second month of lactation and T2 was dry.

The experimental rations were fed twice daily at 8 a.m. and 3 p.m. for at least three weeks before each trial. Alfalfa hay was used for the roughage. The concentrate mixture contained 77% ground corn, 20% soybean oil meal, 1% Ca_2HPO_4, 1% NaCl, 1% $CaCO_3$ plus vitamin A and irradiated yeast. For the restricted roughage trials, T1 received a ration of 28 lb. of concentrate plus 4 lb. of hay and C707 received 10 lb. of concentrate plus 4 lb. of hay. For the all hay trials, T2 received a ration of 30 lb. of hay and C707 received 24 lb. of hay.

On the days of the four trials, feeding was restricted to 2 hours in the morning for the all hay ration and ¾ hour for the high concentrate-

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restricted roughage ration. Water was permitted at will during the next 24 hours. During the feeding period, T1 ate 11 lb. of concentrate and 2 lb. of hay, T2 ate 8 lb. of hay, C707 ate 5 lb. of concentrate and 2 lb. of hay for the restricted roughage trial and 6 lb. of hay for the all hay trial.

The time half way between initiation and termination of feeding was taken as 0 hour for purposes of later sampling and computation. However, in order to avoid disturbing the animal while eating, the sample recorded as 0 hour was taken just prior to feeding. Ten to 11 samples of the liquid in the middle part of the ventral sac in the rumen were taken at intervals up to 24 hours after 0 hour and strained through cheese-cloth into a volumetric flask containing 50% sulfuric acid equal to 3% of its volume. This volume of sulfuric acid lowered the pH to 1.5 to 2.0 and yielded a final sample containing about 91.3% rumen liquid on a dry matter free basis. Most of the suspended solids in the initial sample were precipitated by the sulfuric acid. These samples were stored at 6° C. until analyzed.

Cheese-cloth strained rumen liquid from T1 and T2 were used for inocula for the *in vitro* fermentations which were conducted in 1 litter Erlenmeyer flasks in a 39° C. water bath. Four flasks were pre-flushed with CO_2 and placed in the bath within 20 minutes after removal of the inocula from the cows. Two of the flasks contained 600 gm. of inocula from the hay fed cow. An estimated 12 gm. of total digestible nutrients (T.D.N.) as alfalfa leaf meal served as substrate in one of the flasks and an estimated 12 gm. of T.D.N. from the concentrate mix served as substrate in the other. The other 2 flasks were similarly prepared using 600 gm. of inocula from the concentrate fed cow. The flasks were kept stoppered except when removing samples with a pipette at the beginning of the fermentation and at 4 intervals between 40 and 140 minutes thereafter.

Acetic, propionic, and butyric acids were determined by direct chromatography of rumen liquid on silica gel as described by Harper (1953) with modifications. Thirteen gm. of dry silicic acid and 7.5 ml. of 1.6M potassium phosphate pH 6.3 buffer were used for the buffered section. A 38 mm. diameter column was used. The sulfuric acid section of Harper (1953) was omitted since it had no effect on the results of this experiment. The sample section was prepared by weighing 6 to 7 gm. of rumen liquid directly on to 10 gm. of dry silicic acid on a watch glass. The silicic acid and sample were then quantitatively transferred to a mortar and thoroughly mixed. Fifty ml. of chloroform was

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used to form a slurry and used as a vehicle to transfer this section to the column. Eluate fractions of appropriate size as determined by preliminary trials were collected starting immediately after adding the sample section. The flow rate was about 150 ml. per hour. Four columns were run simultaneously. Further additions of solvent were made using a separatory funnel adjusted to keep 5 to 10 ml. of solvent covering the column. A 50 ml. addition of pure chloroform was followed by 50 ml. portions of 1, 2.5, 5, 10 and 20% n-butanol in chloroform solutions. Additional 20% n-butanol in chloroform was used to give a total eluate volume of 500 ml. Blank determinations were made with each batch of reagents and appropriate corrections made.

The chromatographic procedure was tested with pure acids alone, and added to rumen liquid. Recovery in 4 trials with 0.5 to 3.8 millimoles of added acetic acid was 93% with a standard deviation of 8%. Recovery of 0.2 to 0.5 millimoles of butyric acid was similarly 96±6%. Threshold volumes for butyric, propionic, and acetic acids were about 70, 200, and 280 ml., but varied somewhat with changes in reagents and the amount of short chain acid applied to the column. The values reported as butyric acid include traces of higher acids. For analysis of the volatile acids produced *in vitro*, the method of Keeney (1955) was used with slight modification. Results from the two methods were in good agreement.

Results and Discussion

Figure 1 portrays the regression of the concentrations of acetic, propionic, and butyric acids in the rumen on time after feeding. Table 1 lists the amounts of acetic, propionic, and butyric acids produced from each lb. of T.D.N. ingested. It was assumed for computational purposes that the hay contained 50% T.D.N. and the concentrate 75%.

The values recorded in table 1 were derived from the regression equations portrayed in figure 1 whereby the concentrations of these acids in the rumen were adjusted for: (a) disappearance from the rumen with time after feeding; (b) the portion of the potential concentration of these acids which remained to be produced with time after feeding; (c) the residual concentration of these acids remaining from previous feedings. An example of these calculations is shown in figure 2. The rates at which the acids disappeared from the rumen are listed in table 2.

The natural logarithms of the acetic acid concentrations from 3 hours after feeding, and of the propionic and butyric acid concentrations from 3.5 hours after feeding, through the fasting period were fitted to a regression on time by the method of least squares as described by

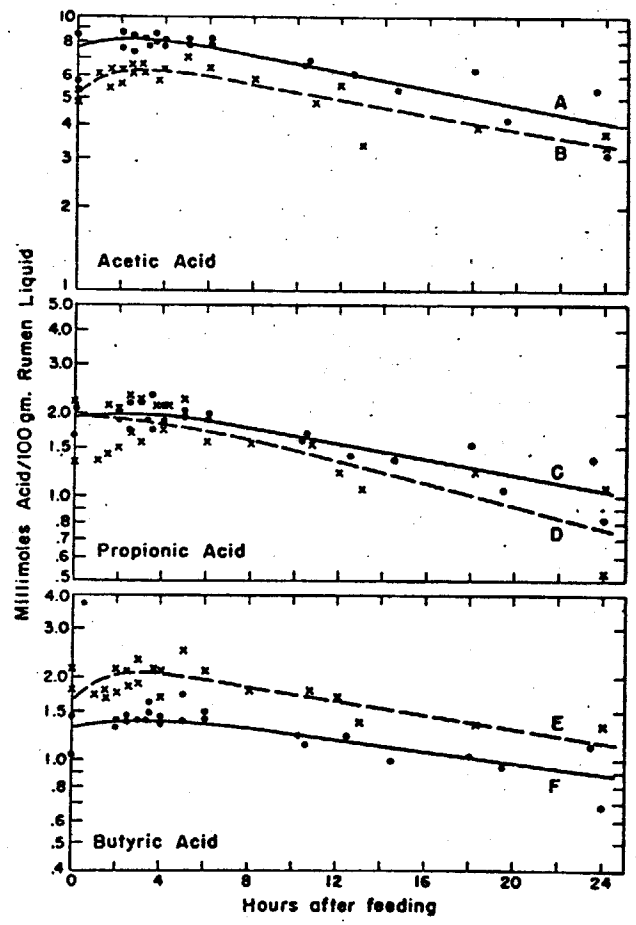

Figure 1. The concentration of short chain acids in the rumen.
Hay ration ———— Concentrate ration — — — —

The equations for the curves are:

A. $A_t = 9.59e^{-0.0234t} - 1.90e^{-0.5231t}$.
B. $7.16e^{-0.0311t} - 2.16e^{-0.5381t}$.
C. $2.28e^{-0.0321t} - 0.36e^{-0.4501t}$.
D. $2.36e^{-0.0450t} - 0.41e^{-0.5701t}$.
E. $2.35e^{-0.0244t} - 0.73e^{-0.7381t}$.
F. $1.63e^{-0.0248t} - 0.32e^{-0.8931t}$.

Snedecor (1946). Any production of these acids subsequent to 3 or 3.5 hours after feeding would result in deviations from their logarithmic disappearance. The small magnitude of the standard errors reported in table 2 largely precludes the possibility.

The 0 hour extrapolation of this regression is the natural logarithm of A_0 and the slope is k_1. The concentration of acetic, propionic, and butyric acids determined between 0 and 3 or 3.5 hours after feeding were subtracted from the A_0 value adjusted for time after feeding according to the disappearance regression. The natural logarithms of

TABLE 1. THE AMOUNT OF ACETIC, PROPIONIC, AND BUTYRIC ACIDS PRODUCED FROM ONE POUND OF TOTAL DIGESTIBLE NUTRIENTS

	All hay ration		Restricted roughage —concentrate ration		Pooled data from both rations
Acid	mM/100 gm.[a]	%	mM/100 gm.[a]	%	mM/100 gm.[b]
Acetic	0.52	68.4	0.30	65.2	.24–.67
Propionic	0.15	19.7	0.06	13.0	.02–.26
Butyric	0.09	11.8	0.10	21.7	.04–.21
Total	0.76	....	0.46	....	.....

[a] Millimoles of acid per 100 gm. of rumen liquid.
[b] Range at 95% confidence level.

these differences were fitted to a regression against time as before. The slope of this second regression equation is k_2 and the 0 hour extrapolate is the natural logarithm of A_p. The tests for significance were carried out on the logarithms.

There were no significant differences at the 5% level between any of the disappearance rates. However, the pooled disappearance rates for acetic acid approached a significantly faster rate than that for the butyric acid. This is in agreement with Pfander and Phillipson (1953). The rates of production of the acids were not significantly different in any case and ranged from 28 to 84% per hour. The amounts of each acid produced from a pound of T.D.N. from each ration were not significantly different. However, acetic acid production from hay approached significantly greater value than that from the high concentrate ration. The molar ratio of acetic to propionic acid was reduced by the high concentrate ration but, the reduction was not as marked as that reported by Tyznik (1951).

The initial concentration of acetic and propionic acids in the *in vitro* fermentations were subtracted from the concentration of these acids

present after incubation for 40 to 140 minutes. These values, presented in table 3, represent the acid produced during this time with the different treatments. Since the fermentations were not complete these

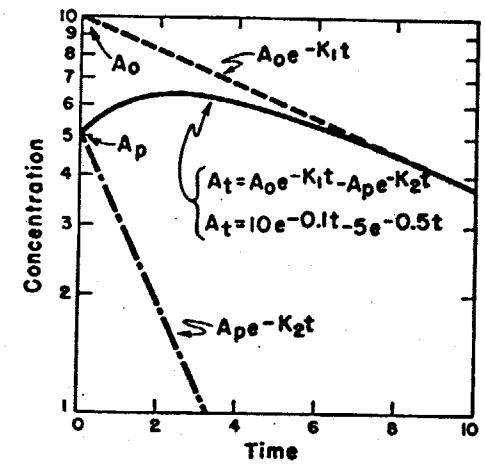

Figure 2. A graphic portrayal of the turnover curve analysis; ——— disappearance of product from the rumen; — — — conversion of feed to product; ———— concentration of product in the rumen resulting from combination of the other two curves.

Where, $A_t = A_o e^{-k_1 t} - A_p e^{-k_2 t}$

A_t = the concentration of the acid in mM per 100 gm. of rumen liquid.

A_o = the disappearance regression extrapolated to 0 time; this could represent the amount of acid which would be present if the fermentation were completed without any disappearance of the acid;

$A_p = (A - A_t)$ when t is 0; this should represent the amount of acid produced from the current feeding in mM/100 gm. of rumen liquid;

e = base of the natural system of logarithms (2.71828);

k_1 = rate of disappearance of the acid from the rumen; 100 k_1 = %/hour of disappearance of the acid present at any given time;

k_2 = rate of acid production of rate of conversion of fermentable feed into its acid product;

t = time after feeding in hours.

values are not representative of the total amount of the acid which could be formed from the substrate. More acetic acid was produced from the hay substrate than from the concentrate substrate regardless of inocula ($P<0.01$). More propionic acid was produced with inocula from the concentrate fed cow than with inocula from the hay fed cow

TABLE 2. THE DISAPPEARANCE RATES FOR ACETIC, PROPIONIC, AND BUTYRIC ACIDS FROM THE RUMEN

	All hay ration		Restricted roughage ration		Pooled data from both rations	
Acid	% per hr.	S.E.[a]	% per hr.	S.E.[a]	% per hr.	S.E.[a]
Acetic	3.5	0.4	3.1	0.5	3.3	0.3
Propionic	3.2	0.5	4.6	0.9	...	...
Butyric	2.5	0.5	2.8	0.5	2.7	0.3

[a] Standard error.

regardless of the substrate being fermented ($P<.01$). This suggests that the rations themselves control the production of acetic acid while the amount of propionic acid produced is controlled by the microbiota.

The energy supplied to the cow by the short chain acids can be computed from the data in table 2. The average animal in the *in vivo* experiment weighed 1270 lb. Carroll and Hungate (1954) concluded that rumen contents constitute 14% of the bovine body weight. The average weight of the rumen contents in this experiment would then be 178 lb. which would contain about 70 kg. of rumen liquid (dry matter free). Computing from the 95% confidence level ranges given in table 1, a lb. of T.D.N. yields 168 to 469 mM. of acetic acid, 14 to 182 mM. propionic acid, and 28 to 147 mM. butyric acid. Converting

TABLE 3. THE FORMATION OF ACETIC AND PROPIONIC ACIDS BY RUMEN MICROBIOTA *IN VITRO*

	Mean production of acids Millimoles of acid per 100 gm. fermentation liquid	
Treatment	Acetic acid	Propionic acid
Hay inoculum-hay substrate	2.15 (0.21)[a]	1.06 (0.07)
Hay inoculum-grain substrate	0.96 (0.40)	0.22 (0.06)
Hay inoculum-mean	1.55 (0.30)[b]	0.64 (0.16)
Grain inoculum-hay substrate	3.35 (0.26)	2.90 (0.25)
Grain inoculum-grain substrate	1.69 (0.20)	2.37 (0.27)
Grain inoculum-mean	2.52 (0.35)	2.64 (0.19)
Difference due to inocula:	0.964[c]	2.00**
Hay substrate-mean	2.75 (0.28)	1.98 (0.37)
Grain substrate-mean	1.33 (0.25)	1.30 (0.43)
Difference due to substrate:	1.42**	0.68

[a] Mean of four samples of fermentation liquid taken after 40 to 140 minutes incubation; the standard deviation of the mean is shown in parentheses.
[b] Mean of eight samples; the standard deviation of the mean is shown in parentheses.
[c] Approaches significance at the 5% level.
** Significant at the 1% level.

the values to kcal., a total energy of 55.0 to 232.3 kcal. per lb. of T.D.N. is obtained, 35.2 to 98.3 are represented by acetic acid, 5.1 to 66.9 by propionic acid, and 14.7 to 77.1 by butyric acid. If one lb. of T.D.N. is equivalent to 1814 Cal. (Brody, 1945), the cow obtains 3 to 13% of its energy from short chain acids.

A constant rumen volume was assumed in computing these regression equations. The data of Chance *et al.* (1953) support this assumption. Irregular fluctuations in volume would increase the standard error and hence, be corrected for by the range in the 3 to 13% estimate. However, if rumen volume increases appreciably and regularly after eating due to salivation, drinking, or influx of water from the tissues, these estimates would be too low. Similarly, if rumen volume decreases appreciably with fasting, as suggested by the data of Weller and Gray (1954), both the disappearance rates and the energy estimates are biased downwards. If a method for determination of rumen volume at frequent intervals were available, the accuracy of these estimates would be greatly enhanced.

The carbon balance studies of McNaught (1951) and of Heald (1952) indicate a 25 to 50% conversion of feed energy to short chain acid energy. Carroll and Hungate (1954) estimate that short chain acids account for 66% of the available feed energy while Pfander and Phillipson (1953) imply a similar value.

Summary

The concentration of acetic, propionic, and butyric acids in 3 bovine rumens was determined at various times after feeding either an all hay ration or one consisting largely of concentrates. Two trials were conducted on each ration. The concentration of each acid first increased and then decreased with time after feeding. The values obtained were fitted to turnover curves. Acetic acid disappeared from the rumen at the rate of 3.3% per hour and butyric acid at the rate of 2.7% per hour. The standard error was 0.3%. The disappearance rate of propionic acid on the high concentrate ration was 4.6% per hour versus 3.2% per hour on the hay ration. The rates for conversion of feed to short chain acids ranged from 28 to 84% per hour.

Pooling data from the 4 trials, the mM of acids per 100 gm. of rumen liquid which were produced from 1 lb. of T.D.N. were, 0.24 to 0.67 acetic, 0.02 to 0.26 propionic, and 0.04 to 0.21 butyric (P .05). The average rumen in these trials was estimated to contain 70 kg. of liquid.

Using these values, it was computed that the cow obtains 3 to 13% of its energy from short chain acids.

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NOV GMENTS

er a grant from the National Science Foundation
author wishes to acknowledge also the advice and
en-Bazire and Professor R. Y. Stanier.

SUMMARY

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ydromonomethoxylycopene and of pigment
thoxylycopen-6-one.

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Alteration of the Pattern of Hepatic Carbohydrate Metabolism by Propionate and Butyrate[1,2]

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INTRODUCTION

In earlier communications from this laboratory (1–3), it was reported that certain short-chain fatty acids are remarkably effective in altering the metabolism of acetate by liver slices. Propionate and butyrate severely reduced the ability of rat liver slices to oxidize acetate-1-C^{14} to $C^{14}O_2$. Moreover, these fatty acids depressed hepatic acetate utilization as a whole, but not as severely as they depressed acetate oxidation. Pennington (4) has recently confirmed our findings on the inhibition of hepatic acetate oxidation by short-chain fatty acids.

The effect of propionate and butyrate on the conversion of acetate to long-chain fatty acids by liver slices was also studied (1–3). Propionate depressed the incorporation of acetate-1-C^{14} into fatty acids. Butyrate failed to reduce the amount of acetate-1-C^{14} incorporated into fatty acids by rat liver slices.

The above evidence indicates that propionate and butyrate alter the pattern of acetyl metabolism in the liver slice. On the assumption that acetyl–coenzyme A (CoA) is the chemical form common to all-*trans*-acetylations in the mammalian cell, it is reasonable to suppose that short-chain fatty acids would affect hepatic acetyl metabolism in the same way irrespective of the source of the acetyl group. To test this

[1] This work was accomplished under Contract U. S. Air Force 41(657)-145 monitored by the Alaskan Air Command, Arctic Aeromedical Laboratory, and with the aid of Research Grant RG 4989, U. S. Public Health Service.

[2] A preliminary report of this work appeared as an abstract in *Federation Proc.* **16**, 104 (1957).

[3] Senior Research Fellow, U. S. Public Health Service.

[4] Predoctoral Fellow of the National Science Foundation.

hypothesis, a study was made of the effects of propionate and butyrate on the metabolism of carbohydrates and carbohydrate intermediates. The results of these experiments are reported below.

Experimental

Adult male rats of the Wistar strain were allowed to eat *ad libitum* for 10 days a diet composed of 25 g. casein, 10 g. fat, 51 g. dextrose, 3 g. salt mix (5), 5 g. cellulose, 6 g. brewer's yeast, 0.04 g. cod liver oil concentrate, and 0.01 g. α-tocopherol. Following this period of dietary stabilization, the animals were killed by a blow on the head, and the livers were rapidly excised and placed in ice-cold Krebs-Henseleit bicarbonate buffer solution (6). Liver slices were prepared by a McIlwain tissue chopper (7). The slices were collected in a Petri dish containing ice-cold buffer. They were then blotted free of excess solution on filter paper, and aliquots of approximately 250 mg. tissue were weighed and transferred to incubation flasks (8). All incubations were carried out in duplicate.

The main compartment of the incubation flasks contained 250 mg. slices, 4.5 ml. of the Krebs-Henseleit bicarbonate buffer, and 0.5 ml. saline containing the labeled substrate at trace concentrations. When unlabeled substrates were also present, they were neutralized to pH 7.4 with sodium hydroxide and dissolved in the buffer. The slices were incubated for 3 hr. at 37.5°C. with continuous shaking.

The total lipides (fatty acids and unsaponifiable materials) were determined by a modification (9) of the method of Baruch and Chaikoff (8) on pooled liver slices from duplicate flasks. An aliquot of the lipide extract (containing the total lipide) was used for the precipitation of cholesterol digitonide using the method of Sperry and Webb (10). The C^{14} content of the lipides and cholesterol digitonide was determined by methods already described (9).

The $C^{14}O_2$ formed during the incubation was recovered by the method of Chernick *et al.* (11) and was counted either in the form of $BaCO_3$ by the method of Entenman *et al.* (12) or by a liquid-counting method consisting of counting a 1-ml. aqueous C^{14} sample with a Nuclear-Chicago D-47 end-window tube in a high humidity chamber.

The C^{14} content of the glucose and lactate used in the experiments was determined by the liquid-counting method.

The unlabeled butyrate and propionate were purchased from commercial sources and were of the highest purity obtainable. Each was further purified by fractional distillation; a Widmer fractionating column was used in these distillations.

Results

Data on the effects of propionate and butyrate on the metabolism of trace levels of uniformly labeled glucose (glucose-E-C^{14}) by rat liver slices are reported in Table I.

Propionate reduced the $C^{14}O_2$ production by the liver slices to about 53% of that produced by the control liver slices, while reducing the incorporation of C^{14} into lipides to 24% of the control value.

of the effects of propionate and butyrate
ydrates and carbohydrate intermediates.
ts are reported below.

XPERIMENTAL

rain were allowed to eat *ad libitum* for 10 days
g. fat, 51 g. dextrose, 3 g. salt mix (5), 5 g. cel-
cod liver oil concentrate, and 0.01 g. α-tocoph-
ary stabilization, the animals were killed by a
ere rapidly excised and placed in ice-cold Krebs-
on (6). Liver slices were prepared by a McIlwain
re collected in a Petri dish containing ice-cold
ee of excess solution on filter paper, and aliquots
were weighed and transferred to incubation
rried out in duplicate.
incubation flasks contained 250 mg. slices, 4.5
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trations. When unlabeled substrates were also
pH 7.4 with sodium hydroxide and dissolved in
ted for 3 hr. at 37.5°C. with continuous shaking.
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and lactate used in the experiments was deter-
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propionate were purchased from commercial
urity obtainable. Each was further purified by
fractionating column was used in these distilla-

RESULTS

ionate and butyrate on the metabolism of
beled glucose (glucose-E-C^{14}) by rat liver
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$^{14}O_2$ production by the liver slices to about
control liver slices, while reducing the in-
s to 24% of the control value.

TABLE I

Effects of Propionate and Butyrate on Hepatic Glucose Metabolism

Number of rats	Unlabeled substrate	concn. × 10^{-3} M	Conversion of glucose-E-C^{14} to[a] CO_2 %	Lipides %
6	None	0	4.0 ± 0.09[b]	0.24 ± 0.057
	Butyrate	4	2.5 ± 0.14	0.07 ± 0.016
	Propionate	4	2.1 ± 0.19	0.06 ± 0.011

[a] Uniformly labeled glucose (glucose-E-C^{14}).
[b] Standard deviation of the mean.

Butyrate also inhibited $C^{14}O_2$ production from the added glucose-E-C^{14}, the $C^{14}O_2$ yield being about 63% of the amount found with slices incubated in the absence of butyrate. Moreover, butyrate inhibited C^{14} incorporation into lipides; the C^{14} content of the lipides was reduced to 29% of that present in control liver slices, this being a far more severe inhibitory effect than that found for $C^{14}O_2$ production. These findings are particularly noteworthy when compared with the effect of butyrate on hepatic acetate metabolism, namely, the severe reduction in $C^{14}O_2$ production with little or no inhibitory effect on the incorporation of acetate-1-C^{14} into fatty acids.

It should be noted that the lipide data of these experiments can properly be compared with fatty acid data of the earlier studies (1–3) because very little glucose-C^{14} is incorporated into cholesterol in liver slices under the conditions used. It is apparent therefore that the C^{14} content of the lipide fraction is composed almost entirely of fatty acids.

The above results suggest that acetyl groups derived from glucose are influenced in a qualitatively different manner by short-chain fatty acids than are those derived from acetate. However, part of the difference in effects noted may result from the fact that the carbons 3 and 4 of glucose (via the glycolytic pathway) and carbon 1 (when it is oxidized via the phosphogluconate pathway) yield CO_2 before an acetyl group is formed. In order to learn more about the effects of propionate and butyrate on the metabolism of acetyl groups derived from glucose, the effect of these compounds on the oxidation of glucose-1-C^{14} and glucose-6-C^{14} was studied (Table II). Unfortunately, not enough radioactivity was present to permit lipogenesis studies from these compounds. The striking fact, however, is that butyrate and propionate reduced $C^{14}O_2$ production from glucose-1-C^{14} and glucose-6-C^{14} to about the same extent as they reduced it from glucose-E-C^{14}. When one further considers that glucose-6-C^{14} must form an acetyl group on its way to $C^{14}O_2$, we must

TABLE II

Effects of Propionate and Butyrate on $C^{14}O_2$ Recoveries from Glucose-1-C^{14} and Glucose-6-C^{14}

Number of rats	Unlabeled substrate	concn. × 10^{-3} M	Conversion of Glucose-1-C^{14} to CO_2 %	Conversion of Glucose-6-C^{14} to CO_2 %
8	None	0	3.3 ± 0.40[a]	1.9 ± 0.18
	Butyrate	4	2.2 ± 0.32	1.4 ± 0.17
6	None	0	3.5 ± 0.55	2.0 ± 0.31
	Propionate	4	2.4 ± 0.36	1.0 ± 0.16

[a] Standard deviation of the mean.

conclude that the oxidation of acetyl groups from glucose are not greatly inhibited by the butyrate and propionate.

Since the above results indicate that propionate and butyrate influence the metabolism of glucose and acetate in different ways, a study was made of these two fatty acids on lactate metabolism. Since lactate-2-C^{14} gives rise to an acetyl group labeled in the one position, this enables us

TABLE III

Effects of Propionate and Butyrate on the Metabolism of Lactate-2-C^{14} by Rat Liver Slices

Expt. No.	Unlabeled substrate	concn. × 10^{-3} M	Conversion of lactate-2-C^{14} to: CO_2 %	Lipides %
1	None	0	26	0.6
	Butyrate	4	16	0.3
	Propionate	4	16	0.2
2	None	0	29	0.7
	Butyrate	4	19	0.3
	Propionate	4	17	0.2
3	None	0	22	2.5
	Butyrate	4	14	1.0
	Propionate	4	14	0.7
4	None	0	24	3.1
	Butyrate	4	14	0.6
	Propionate	4	17	1.4
5	None	0	22	0.2
	Butyrate	4	17	0.1
	Propionate	4	14	0.0

TABLE II

d Butyrate on $C^{14}O_2$ Recoveries from -C^{14} and Glucose-6-C^{14}

ncn. × 10^{-3} M	Conversion of Glucose-1-C^{14} to CO_2 %	Conversion of Glucose-6-C^{14} to CO_2 %
0	3.3 ± 0.40[a]	1.9 ± 0.18
4	2.2 ± 0.32	1.4 ± 0.17
0	3.5 ± 0.55	2.0 ± 0.31
4	2.4 ± 0.36	1.0 ± 0.16

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cetyl groups from glucose are not greatly propionate.

te that propionate and butyrate influence acetate in different ways, a study was n lactate metabolism. Since lactate-2-C^{14} beled in the one position, this enables us

TABLE III

nd Butyrate on the Metabolism of ^{14} by Liver Slices

te 10^{-3} M	Conversion of lactate-2-C^{14} to: CO_2 %	Lipides %
0	26	0.6
4	16	0.3
4	16	0.2
0	29	0.7
4	19	0.3
4	17	0.2
0	22	2.5
4	14	1.0
4	14	0.7
0	24	3.1
4	14	0.6
4	17	1.4
0	22	0.2
4	17	0.1
4	14	0.0

TABLE IV

Effects of Propionate and Butyrate on $C^{14}O_2$ Recoveries from Lactate-1-C^{14} by Rat Liver Slices

Number of rats	Unlabeled substrate	concn. × 10^{-3} M	Conversion of lactate-1-C^{14} to CO_2 %
3	None	0	54 ± 1.7[a]
	Butyrate	4	50 ± 3.4
	Propionate	4	39 ± 3.0

[a] Standard deviation of the mean.

directly to compare the effect of butyrate and propionate on the metabolism of the acetyl groups formed from a carbohydrate intermediate with those formed from acetate-1-C^{14}. The results are recorded in Table III. It is apparent that propionate and butyrate inhibit C^{14} incorporation into lipides much more than they reduce $C^{14}O_2$ production from lactate-2-C^{14}. These results are identical with those found in the case of glucose and again contrast sharply with the results previously reported for acetate-1-C^{14} (1–3). In some of these experiments the C^{14} incorporation into cholesterol and fatty acids was determined separately; the per cent inhibition of lactate-2-C^{14} recovered in cholesterol was of the same order of magnitude as the inhibition of incorporation into fatty acids.

The effect of the above fatty acids on lactate-1-C^{14} oxidation was also studied, and the results are recorded in Table IV. Butyrate has practically no effect while propionate definitely depresses $C^{14}O_2$ production from lactate-1-C^{14}. It is apparent that the action of butyrate on lactate-2-C^{14} metabolism must have been at the acetyl level, or the Krebs cycle, or both.

Discussion

In our earlier studies (1–3), it was established that propionate and butyrate greatly reduce the hepatic $C^{14}O_2$ production from acetate, while the C^{14} incorporation into fatty acids is inhibited by propionate to a lesser extent, and by butyrate not at all.

In the present study, we have also found that propionate and butyrate cause large changes in the pattern of hepatic glucose and lactate metabolism. However, these alterations are of a qualitatively different nature from those found in the case of acetate metabolism. Both butyrate and propionate depress the incorporation of glucose-C^{14} and lactate-2-C^{14} into lipides far more than they reduce $C^{14}O_2$ production from these compounds.

Consideration of the effects of butyrate on hepatic metabolism leads one to conclude that the way in which butyrate alters hepatic acetyl metabolism depends upon the source of the acetyl group. Strong evidence for such a conclusion lies in the comparison of the effects of butyrate on hepatic acetate-1-C^{14} metabolism and hepatic lactate-2-C^{14} metabolism. Presumably both of these compounds must form acetyl-CoA before the labeled carbon can be converted to either $C^{14}O_2$ or lipide-C^{14}; yet butyrate acts to inhibit $C^{14}O_2$ production from acetate-1-C^{14} without reducing lipide-C^{14} incorporation, while under the same conditions butyrate severely reduces the incorporation of lactate-2-C^{14} into lipide-C^{14} without greatly affecting the $C^{14}O_2$ production.

Although the data are more equivocal, a similar conclusion may be drawn concerning the actions of propionate on hepatic acetyl metabolism.

The above findings can be interpreted mechanistically in at least two ways. It is possible that acetate and carbohydrate form two or more chemically distinct "active acetate" groups. For example, the acetyl-adenosine monophosphate (AMP) compound of Berg (13) may only be formed from acetate; the presence of butyrate may alter the metabolic priorities of acetyl-AMP, possibly enhancing its conversion into long-chain fatty acids and preventing its conversion to acetyl-CoA and thence to CO_2. An alternative explanation—but not necessarily the only one—lies in the possibility of two or more intracellular sites of acetyl formation, thereby making chemically identical but functionally quite distinct acetyl groups available to the cell.

Summary

Butyrate and propionate caused a reduction in $C^{14}O_2$ and lipide-C^{14} production from evenly labeled glucose, glucose-1-C^{14}, glucose-6-C^{14}, and lactate-2-C^{14}. Unlike the action of these acids on acetate metabolism, the inhibition of lipogenesis from glucose and lactate was more severe than the reduction in oxidation of these compounds. These striking differences between the inhibitory actions of butyrate and propionate on hepatic acetate metabolism on the one hand and hepatic carbohydrate metabolism on the other seem to suggest that the metabolic path of the C-2 fragment derived from these two sources must differ.

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Summary

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icids.

in a slight excess of nitric acid
w precipitate forms.
g with 5 ml. of diluted hydro-
1 ml. of ammonia T.S., drop-
of ammonium oxalate T.S. A

of 90.0 percent of $Ca_3(PO_4)_2$,

ercent.

parts per million (0.0003 per-

r million (0.005 percent).
an 3 ts per million (0.003

ion (0.0005 percent).

g., and dissolve it in a mixture
d nitric acid T.S. Filter, if
ficient ammonia T.S. to the
en dissolve the precipitate by
T.S. Adjust the temperature
molybdate T.S., and maintain
minutes, stirring occasionally.
water by decantation, using
he precipitate to a filter, and
n 100) until the last washing
e precipitate and filter to the
odium hydroxide, agitate until
of phenolphthalein T.S., and
ifuric acid. Each ml. of 1 *N*
. of $Ca_3(PO_4)_2$.
about 2 grams, and dissolve,
oric acid. Cool, add 1 or 2
rate the excess of 1 *N* hydro-
to a yellow color, vigorously
Not less than 12.5 ml. and
ric acid is consumed for each
s.

105a

Loss on ignition. Weigh accurately about 1 gram, and ignite, preferably in a muffle furnace, at 800° to 825° to constant weight.

Arsenic. A solution of 1 gram in 25 ml. of diluted hydrochloric acid T.S. meets the requirements of the *Arsenic Test*, page 865.

Fluoride. Weigh accurately 1.0 gram, and proceed as directed in the *Fluoride Limit Test*, page 917.

Heavy metals. Warm 1.33 grams with 7 ml. of diluted hydrochloric acid T.S. until no more dissolves, dilute to 50 ml. with water, and filter. A 25-ml. portion of the filtrate meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*). (*Note:* Filter the mixture after pH adjustment.)

Lead. A solution of 250 mg. in 5 ml. of diluted hydrochloric acid T.S. meets the requirements of the *Lead Limit Test*, page 929, using 1.25 mcg. of lead ion (Pb) in the control.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Anticaking agent; buffer; nutrient; dietary supplement.

CALCIUM PROPIONATE

$(CH_3CH_2COO)_2Ca$

$C_6H_{10}CaO_4$ Mol. wt. 186.22

DESCRIPTION

White crystals or crystalline solid, possessing not more than a faint odor of propionic acid. One gram dissolves in about 3 ml. of water. The pH of a 1 in 10 solution is between 8 and 10.

IDENTIFICATION

A. A 1 in 20 solution gives positive tests for *Calcium*, page 926.

B. Upon ignition at a relatively low temperature, it yields an alkaline residue which effervesces with acids.

C. Warm a small sample with sulfuric acid. Propionic acid, recognizable by its odor, is evolved.

SPECIFICATIONS

Assay. Not less than 98.0 percent of $C_6H_{10}CaO_4$, calculated on the anhydrous basis.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).
Fluoride. Not more than 30 parts per million (0.003 percent).
Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Insoluble substances. Not more than 0.2 percent.
Magnesium (as MgO). Passes test (about 0.4 percent).
Water. Not more than 5 percent.

TESTS

Assay. Dissolve about 400 mg., accurately weighed, in 100 ml. of water. While stirring, preferably with a magnetic stirrer, add about 30 ml. of 0.05 *M* disodium ethylenediaminetetraacetate from a 50-ml. buret, then add 15 ml. of sodium hydroxide T.S. and 300 mg. of hydroxy naphthol blue indicator, and continue the titration to a blue end-point. Each ml. of 0.05 *M* disodium ethylenediaminetetraacetate is equivalent to 9.311 mg. of $C_6H_{10}CaO_4$.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Fluoride. Proceed as directed in the *Fluoride Limit Test*, page 917, using *Method I* (1.67-gram sample) or *Method III* (1.0-gram sample).

Heavy metals. A solution of 2 grams in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Insoluble substances. Dissolve 10 grams in 100 ml. of hot water, filter through a tared filtering crucible, wash the insoluble residue with hot water, and dry at 105° to constant weight.

Magnesium. Place 400.0 mg. of the sample, 5 ml. of diluted hydrochloric acid T.S., and about 10 ml. of water in a small beaker, and dissolve the sample by heating on a hot plate. Evaporate the solution to a volume of about 2 ml., and cool. Transfer the residual liquid into a 100-ml. volumetric flask, dilute to volume with water, and mix. Dilute 7.5 ml. of this solution to 20 ml. with water, add 2 ml. of sodium hydroxide T.S. and 0.05 ml. of a 1 in 1000 solution of Titan yellow (Clayton yellow), mix, allow to stand for 10 minutes, and shake. Any color does not exceed that produced by 1.0 ml. of *Magnesium Standard Solution* (50 mcg. Mg ion) in the same volume of a control containing 2.5 ml. of the sample solution (10-mg. sample) and the quantities of the reagents used in the test.

Water. Determine by the *Karl Fischer Titrimetric Method*, page 977.

Packaging and storage. Store in tight containers.

Functional use in foods. Preservative; mold and rope inhibitor.

an appropriate correction factor. (*Note:* In the calculation of diethyl succinate content, the area and weight percent may be assumed to be identical.) Determine the calibration factor for diethyl maleate by analyzing a sample of known composition to which measured amounts of diethyl maleate have been added, following the procedure described above.

Acidity. Transfer 57 ml. (59 grams) into a 250-ml. Erlenmeyer flask, add phenolphthalein T.S., and titrate with 0.1 *N* alcoholic potassium hydroxide to a pink end-point that persists for at least 15 seconds. Each ml. of 0.1 *N* alcoholic potassium hydroxide is equivalent to 5.904 mg. of succinic acid ($C_4H_6O_4$).

Diethyl maleate. Determine as directed under *Assay and limit of diethyl maleate.*

Heavy metals. Prepare and test a 500-mg. sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Water. Determine as directed in the *Karl Fischer Titrimetric Method*, page 977.

Packaging and storage. Store in tight containers.

Functional use in foods. Flavoring agent.

DILAURYL THIODIPROPIONATE

$(C_{12}H_{25}OOCCH_2CH_2)_2S$

$C_{30}H_{58}O_4S$ Mol. wt. 514.85

DESCRIPTION

White crystalline flakes having a characteristic sweetish, ester-like odor. It is insoluble in water, but soluble in most organic solvents.

SPECIFICATIONS

Assay. Not less than 99.0 percent of $C_{30}H_{58}O_4S$.

Solidification point. Not below 40°.

Limits of Impurities

Acidity (as thiodipropionic acid). Not more than 0.2 percent of $C_6H_{10}O_4S$.

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).

Lead. Not more than 10 parts per million (0.001 percent).

TESTS

Assay. Transfer about 700 mg., accurately weighed, into a 250-ml. Erlenmeyer flask, and add 100 ml. of acetic acid and 50 ml. of alcohol.

Heat the mixtur
pletely dissolved
ethoxychrysoidi
bromine. Wher
drops of the indi
color change fro
(see page 2) an
bromine is equiv
of thiodipropion
subtract this v
calculated from
$C_{30}H_{58}O_4S$.

Solidification
dure, page 954.

Acidity (as thi
weighed, into a 25
of a mixture comp
add 5 drops of p
potassium hydrox
is equivalent to 8.

Arsenic. A *S*
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Heavy metals.
Method II under t
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Lead. A *Samp*
meets the requiren
lead ion (Pb) in th

Packaging and s
Functional use i

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DESCRIPTION

The volatile oil
dried fruit (or seed
grown in various E
yellow liquid with a
fixed oils and in mi
propylene glycol, bu

SPECIFICATIONS

Assay. Not less t
volume, of ketones

Vote: In the calculation of diethyl
:ht percent may be assumed to be
ion factor for diethyl maleate by
:ition to which measured amounts
following the procedure described

:ams) into a 250-ml. Erlenmeyer
titrate with 0.1 *N* alcoholic potas-
nat persists for at least 15 seconds.
n hydroxide is equivalent to 5.904

directed under *Assay and limit of*

: a 500-mg. sample as directed in
:st, page 920, using 20 mcg. of lead

ne *Karl Fischer Titrimetric Method,*

tight containers.
ng agent.

IPROPIONATE

$H_2CH_2)_2S$

Mol. wt. 514.85

characteristic sweetish, ester-like
uble in most organic solvents.

$C_{30}H_{58}O_4S$.
?.

Not more than 0.2 percent of

parts per million (0.0003 percent).
than 20 parts per million (0.002

million (0.001 percent).

:curately weighed, into a 250-ml.
acetic acid and 50 ml. of alcohol.

Heat the mixture at a temperature of about 40° until the sample is completely dissolved, then add 3 ml. of hydrochloric acid and 4 drops of *p*-ethoxychrysoidin T.S., and immediately titrate the solution with 0.1 *N* bromine. When the end-point is approached (pink color), add 4 more drops of the indicator solution and continue the titration, dropwise, to a color change from red to pale yellow. Perform a blank determination (see page 2) and make any necessary correction. Each ml. of 0.1 *N* bromine is equivalent to 25.74 mg. of $C_{30}H_{58}O_4S$. Multiply the percent of thiodipropionic acid, determined in the *Acidity* test, by 2.89, and subtract this value from the percent of dilauryl thiodipropionate calculated from the titration. The difference is the percent purity of $C_{30}H_{58}O_4S$.

Solidification point. Determine as directed in the general procedure, page 954.

Acidity (as thiodipropionic acid). Transfer about 2 grams, accurately weighed, into a 250-ml. Erlenmeyer flask. Dissolve the sample in 50 ml. of a mixture composed of 1 part of methyl alcohol and 3 parts of benzene, add 5 drops of phenolphthalein T.S., and titrate with 0.1 *N* alcoholic potassium hydroxide. Each ml. of 0.1 *N* alcoholic potassium hydroxide is equivalent to 8.91 mg. of $C_6H_{10}O_4S$.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 1-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead on (Pb) in the control (*Solution A*).

Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Packaging and storage. Store in well-closed containers.
Functional use in foods. Antioxidant.

DILL SEED OIL, EUROPEAN

DESCRIPTION

The volatile oil obtained by steam distillation from the crushed, dried fruit (or seeds) of *Anethum graveolens*, L. (Fam. *Umbelliferae*), grown in various European countries. It is a slightly yellowish to light yellow liquid with a caraway-like odor and flavor. It is soluble in most fixed oils and in mineral oil. It is soluble, with slight opalescence, in propylene glycol, but it is practically insoluble in glycerin.

SPECIFICATIONS

Assay. Not less than 42 percent and not more than 60 percent, by volume, of ketones as carvone.

Distillation range. Distil as directed in the general method, page 890.

Specific gravity. Determine by any reliable method (see page 5).

Acidity. Transfer 50 ml. of methanol into a 250-ml. Erlenmeyer flask through which a gentle stream of nitrogen has previously been passed for 2 minutes. Add phenolphthalein T.S. to the methanol and neutralize it with 0.02 *N* alcoholic potassium hydroxide. Introduce 20 ml. (16 grams) of the sample using a suitable transfer pipet. Add 3 or 4 additional drops of phenolphthalein T.S., and titrate with 0.1 *N* sodium hydroxide to a pink end-point that persists for at least 15 seconds. During the titration direct a gentle stream of nitrogen into the flask through a short piece of 6-mm. glass tubing fastened near the tip of the buret. Each ml. of 0.1 *N* sodium hydroxide is equivalent to 7.41 mg. of $C_3H_6O_2$.

Heavy metals. Prepare and test a 500-mg. sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Water. Determine by the *Karl Fischer Titrimetric Method*, page 977, using freshly distilled pyridine instead of methanol as the solvent.

Packaging and storage. Store in tight containers.

Functional use in foods. Flavoring agent.

PROPIONIC ACID

CH_3CH_2COOH

$C_3H_6O_2$ Mol. wt. 74.08

DESCRIPTION

An oily liquid having a slightly pungent, rancid odor. It is miscible with water and with alcohol and various other organic solvents.

SPECIFICATIONS

Assay. Not less than 99.5 percent of $C_3H_6O_2$.

Distillation range. Between 138.5° and 142.5°.

Specific gravity. Between 0.993 and 0.997 at 20°/20°.

Limits of Impurities

Aldehydes (as propionaldehyde). Passes test (limit about 0.05 percent).

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Nonvolatile residue. Not more than 0.01 percent.

Readily oxidiz
about 0.05 perc
Water. Not m

TESTS

Assay. Mix al
recently boiled an
phenolphthalein T
first appearance of
seconds. Each m
mg. of $C_3H_6O_2$.

Distillation ra
dure, page 890.

Specific gravit

Aldehydes. T
stoppered Erlenm
a 1 in 8 solution o
orously. Allow th
0.1 *N* iodine to th
blank treated with
2). The differenc
the blank and that

Arsenic. A *Sa*
pounds meets the

Heavy metals.
requirements of th
ion (Pb) in the con

Nonvolatile r
platinum evapora
weight, and evapo
the dish at 105° fo
tor, and weigh.

Readily oxidiz
hydroxide in 50 ml
fect complete solu
25.0 ml. of this s
flask containing 10
sodium acetate an
minutes, add 5 ml
of hydrochloric ac
the disappearance
(see page 2). Th
sulfate required fo
more than 2.2 ml.

Water. Deter
977.

directed in the general method, page

y any reliable method (see page 5).
ethanol into a 250-ml. Erlenmeyer
m of nitrogen has previously been
phthalein T.S. to the methanol and
potassium hydroxide. Introduce 20
a suitable transfer pipet. Add 3 or
alein T.S., and titrate with 0.1 *N*
int that persists for at least 15 sec-
gentle stream of nitrogen into the
glass tubing fastened near the tip
ium hydroxide is equivalent to 7.41

st a 5.0-mg. sample as directed in
est, page 920, using 20 mcg. of lead

ischer Titrimetric Method, page 977,
ad o thanol as the solvent.

tight containers.
ing agent.

C ACID

COOH

Mol. wt. 74.08

gent, rancid odor. It is miscible
ous other organic solvents.

$C_3H_6O_2$.
and 142.5°.
d 0.997 at 20°/20°.

Passes test (limit about 0.05 per-

3 parts per million (0.0003 per-

than 10 parts per million (0.001

an (percent.

Readily oxidizable substances (as formic acid). Passes test (limit about 0.05 percent).

Water. Not more than 0.15 percent.

TESTS

Assay. Mix about 1.5 grams, accurately weighed, with 100 ml. of recently boiled and cooled water in a 250-ml. Erlenmeyer flask, add phenolphthalein T.S., and titrate with 0.5 *N* sodium hydroxide to the first appearance of a faint pink end-point which persists for at least 30 seconds. Each ml. of 0.5 *N* sodium hydroxide is equivalent to 37.04 mg. of $C_3H_6O_2$.

Distillation range. Determine as directed in the general procedure, page 890.

Specific gravity. Determine by any reliable method (see page 5).

Aldehydes. Transfer 10.0 ml. of the sample into a 250-ml. glass-stoppered Erlenmeyer flask containing 50 ml. of water and 10.0 ml. of a 1 in 8 solution of sodium bisulfite, stopper the flask, and shake vigorously. Allow the mixture to stand for 30 minutes, then titrate with 0.1 *N* iodine to the same brownish yellow end-point obtained with a blank treated with the same quantities of the same reagents (see page 2). The difference between the volume of 0.1 *N* iodine required for the blank and that required for the sample is not more than 1.75 ml.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. A solution of 2 grams in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Nonvolatile residue. Transfer 100 ml. into a tared 125-ml. platinum evaporating dish, previously heated at 105° to constant weight, and evaporate the sample to dryness on a steam bath. Heat the dish at 105° for 30 minutes or to constant weight, cool in a desiccator, and weigh.

Readily oxidizable substances. Dissolve 15 grams of sodium hydroxide in 50 ml. of water, cool, add 6 ml. of bromine, stirring to effect complete solution, and dilute to 2000 ml. with water. Transfer 25.0 ml. of this solution into a 250-ml. glass-stoppered Erlenmeyer flask containing 100 ml. of water, and add 10 ml. of a 1 in 5 solution of sodium acetate and 10.0 ml. of the sample. Allow to stand for 15 minutes, add 5 ml. of a 1 in 4 solution of potassium iodide and 10 ml. of hydrochloric acid, and titrate with 0.1 *N* sodium thiosulfate just to the disappearance of the brown color. Perform a blank determination (see page 2). The difference between the volume of 0.1 *N* sodium thiosulfate required for the blank and that required for the sample is not more than 2.2 ml.

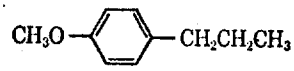
Water. Determine by the *Karl Fischer Titrimetric Method*, page 977.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Preservative; mold and rope inhibitor.

p-PROPYL ANISOLE

Dihydroanethole



$C_{10}H_{14}O$ Mol. wt. 150.22

DESCRIPTION

A colorless to pale yellow liquid having a characteristic anise-type odor with a background of sassafras. It is soluble in most fixed oils and in mineral oil, but is insoluble in glycerin and in propylene glycol.

SPECIFICATIONS

Refractive index. Between 1.502 and 1.506 at 20°.

Solubility in alcohol. Passes test.

Specific gravity. Between 0.940 and 0.943.

TESTS

Refractive index, page 945. Determine with an Abbé or other refractometer of equal or greater accuracy.

Solubility in alcohol. Proceed as directed in the general method, page 899. One ml. dissolves in 5 ml. of 80 percent alcohol and remains in solution upon further dilution.

Specific gravity. Determine by any reliable method (see page 5).

Packaging and storage. Store in tight, full containers in a cool place protected from light.

Functional use in foods. Flavoring agent.

PROPYLENE GLYCOL

1,2-Propanediol; 1,2-Dihydroxypropane; Methyl Glycol

$CH_3.CH(OH).CH_2OH$

$C_3H_8O_2$ Mol. wt. 76.10

DESCRIPTION

A clear, colorless, viscous liquid having a slight, characteristic taste.

It is practically o
air. It is miscibl
tions. It is solubl
immiscible with fix

IDENTIFICATIC

A. Mix 500 m
ml. of pyridine, an
1 hour. Cool, diss
well with 100 mg.
to about 50 ml., an
off the crystals, rec
dry in a current of
(see page 931).

B. Heat gently
odor is evolved, an
acrid odor of acrolei

SPECIFICATIONS

Assay. Not less th
Distillation range.
Specific gravity.
Limits of Impurit
Acidity. Passes
Arsenic (as As).
Heavy metals (a
percent).
Residue on igniti
Water. Not more

TESTS

Assay. Transfer
accurately weighed b
water, and mix. Tra
meyer flask, add 5.0
15 minutes. Add 10
followed by 15.0 ml.
iodide solution (1 in 2
that at the end-point
with 0.1 *N* iodine, usi
to a faint yellow colo
and make any necessa
lent to 3.805 mg. of C

Distillation range
dure, page 890.

Specific gravity.

gram, and proceed as directed

ms in 25 ml. of water meets the
, page 920, using 20 mcg. of lead

0 grams in 100 ml. of hot water,
ole (not glass). Wash the insolu-
for 2 hours, cool, and weigh.

ell-closed containers.
mulsifier; nutrient; dietary sup-

JM TARTRATE

Salt

H).COONa.$4H_2O$

Mol. wt. 282.23

of L(+)-tartaric acid. It occurs
alline powder, having a cooling,
warm dry air, the crystals are
L solves in 1 ml. of water.

r of burning sugar and leaves a
which effervesces with acids.
d 10 ml. of acetic acid. A white
n 15 minutes.
tests for *Tartrate*, page 928.

nd not more than the equivalent

percent.

arts per million (0.0003 percent).
than 10 parts per million (0.001

grams, and proceed as directed

under *Alkali Salts of Organic Acids Assay*, page 864. Each ml. of 0.5 *N* sulfuric acid is equivalent to 70.55 mg. of $C_4H_4KNaO_6 \cdot 4H_2O$.

Water. Determine by the *Karl Fischer Titrimetric Method*, page 977, using a 200-mg. sample and 35 ml. of methanol in the *Procedure*.

Alkalinity. A 1 in 20 solution is alkaline to litmus, but after the addition of 0.2 ml. of 0.1 *N* sulfuric acid to 10 ml. of this solution no pink color is produced by the addition of 1 drop of phenolphthalein T.S.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 2-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Packaging and storage. Store in tight containers.
Functional use in foods. Buffer; sequestrant.

SODIUM PROPIONATE

CH_3CH_2COONa

$C_3H_5NaO_2$ Mol. wt. 96.06

DESCRIPTION

Colorless, transparent crystals or a granular crystalline powder. It is odorless, or has a faint acetic-butyric odor. It is deliquescent in moist air. One gram is soluble in about 1 ml. of water at 25°, in about 0.65 ml. of boiling water, and in about 24 ml. of alcohol. The pH of a 1 in 10 solution is between 8.0 and 10.5.

IDENTIFICATION

A. A 1 in 20 solution gives positive tests for *Sodium*, page 928.

B. Upon ignition, it yields an alkaline residue which effervesces with acids.

C. Warm a small sample with sulfuric acid. Propionic acid, recognized by its odor, is evolved.

SPECIFICATIONS

Assay. Not less than 99.0 percent of $C_3H_5NaO_2$ after drying.

Limits of Impurities

Alkalinity (as Na_2CO_3). Passes test (about 0.15 percent).
Arsenic (as As). Not more than 3 parts per million (0.0003 percent).
Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).
Iron. Not more than 30 parts per million (0.003 percent).
Water. Not more than 1 percent.

TESTS

Assay. Weigh accurately about 250 mg., previously dried at 105° for 1 hour, and dissolve it in 40 ml. of glacial acetic acid, warming if necessary to effect solution. Cool to room temperature, add 2 drops of crystal violet T.S., and titrate with 0.1 *N* perchloric acid. Perform a blank determination (see page 2) and make any necessary correction. Each ml. of 0.1 *N* perchloric acid is equivalent to 9.606 mg. of $C_3H_5NaO_2$.

Alkalinity. Dissolve 4 grams in 20 ml. of water, and add 3 drops of phenolphthalein T.S. If a pink color is produced, not more than 0.6 ml. of 0.1 *N* sulfuric acid is required to discharge it.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. A solution of 2 grams in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Iron. Dissolve 300 mg. in 40 ml. of water, and add 2 ml. of hydrochloric acid, about 40 mg. of ammonium persulfate, and 10 ml. of ammonium thiocyanate T.S. Any red or pink color does not exceed that produced by 0.9 ml. of *Iron Standard Solution* (9 mcg. Fe) in an equal volume of solution containing the quantities of reagents used in the test.

Water. Determine by the *Karl Fischer Titrimetric Method*, page 977.

Packaging and storage. Store in tight containers.

Functional use in foods. Preservative; mold and rope inhibitor.

SODIUM PYROPHOSPHATE

Tetrasodium Diphosphate; Tetrasodium Pyrophosphate

$Na_4P_2O_7$ Mol. wt. 265.90

DESCRIPTION

Sodium pyrophosphate is anhydrous or contains 10 molecules of water of hydration. It occurs as a white, crystalline or granular powder. The decahydrate effloresces slightly in dry air. It is soluble in water, but is insoluble in alcohol. The pH of a 1 in 100 solution is about 10.

IDENTIFICATION

A. A 1 in 20 solution gives positive tests for *Sodium*, page 928.

B. To 1 ml. of a 1 in 100 solution add a few drops of silver nitrate T.S. A white or slightly yellow precipitate is formed which is soluble in diluted nitric acid T.S.

SPECIFICATI

Assay. Not le
basis.

Loss on igniti
$Na_4P_2O_7 \cdot 10H_2O$

Limits of Imp

Arsenic (as
cent).

Fluoride. N

Heavy meta
percent).

Insoluble su

TESTS

Assay. Diss
equivalent to 5
400-ml. beaker.
chloric acid, usi
zinc sulfate (12
1000 ml., filter
minutes. Titra
until a pH of 3
droxide near th
zinc hydroxide
equivalent to 1

Loss on ign
about 800° for

Arsenic. A
ments of the *A*

Fluoride.
in the *Fluoride*

Heavy meta
requirements o
ion (Pb) in the

Insoluble su
and filter throu
with hot water,

Packaging an

Functional us
plement.

123a

Gas Chromatographic Determination of Propionic, Sorbic, and Benzoic Acids in Rye Bread and Margarine

By A. GRAVELAND (Institute for Cereals, Flour and Bread TNO, Wageningen, The Netherlands)

A rapid and accurate method is presented for the GLC determination of propionic and sorbic acids in bakery products and benzoic acid in margarine. The acids are extracted with ether containing orthophosphoric acid, to which valeric acid is added as internal standard. The extract is injected directly into the gas chromatograph. Glass columns (2 m × 2.0 mm id) containing 5% Carbowax 20M-terephthalic acid coated on Chromosorb W (AW-DMCS) are used for analysis. The simplicity and convenience of the method make it suitable for routine determinations.

Low molecular weight carboxylic acids are frequently occurring constituents of food products. They may be inherent to the product, because they are present in the raw materials or formed in the course of the manufacturing process, or they may have been added as a preservative to prevent the development of mold and bacteria.

Because of the use of these acids as preservatives it is desirable to have a rapid method at hand to examine products for their presence. Until recently, routine checking was impossible, as the acids had to be isolated from the materials by steam distillation and recovered as the sodium salts; then they were determined with rather cumbersome methods. The introduction of thin layer chromatographic techniques for the separation and identification of these acids (1, 2) was a step forward. These procedures, however, are also cumbersome and lack the desirable sensitivity.

The newly developed liquid phases for gas chromatography have now made it possible to make a qualitative and quantitative determination of the low molecular weight carboxylic acids within a short time. Complete resolution of all C_1 to C_5 normal and *iso*-acids was attained by Jackson (3), using a sebacic acid column as the liquid phase. The C_2 to C_{11} acids, including some unsaturated and branched isomers, were separated by Nikelly (4) on a column filled with glass microbeads as the solid phase and a mixture of Carbowax and isophthalic acid as the liquid phase. Nieuwenhof and Hup (5) determined free fatty acids (C_2 to C_{18}) by using a Carbowax 20M-terephthalic acid column. We found this column to be equally suitable for the estimation of acetic, propionic, and sorbic acids in rye bread and benzoic acid in margarine.

METHOD

Gas Chromatography

A gas chromatograph (Fractovap Model GV, Carlo Erba Co.) with a flame ionization detector was used; the carrier gas was nitrogen. Glass columns (U-shaped, 2 m × 2 mm id) were packed with 5% Carbowax 20M-terephthalic acid (Hewlett-Packard) coated on 60–80 mesh Chromosorb W (acid-washed, DMCS-treated). Column packing was prepared by dissolving the liquid phase in ethylene chloride, adding the solution to the solid support, and evaporating the volatile solvent in an evaporator. The material was packed into the column by attaching a vacuum pump to one end and vigorously tapping the tube until no more material was accepted. Column ends were plugged with glass wool. A Honeywell recorder (1 mv span) with an Infortronics automatic digital integrator Model CRS-204 was employed for the recording of the components. Operating conditions: column temperature programmed from 100 to 210°C at 5°/min; injection port 260°C; detector 270°C; carrier gas 65 ml/min.

Reagents

(a) *Acids.*—Analytical grade acetic, propionic, butyric, isobutyric, valeric, isovaleric, sorbic, and benzoic acids (Fluka, Basel, Switzerland).

(b) *Internal standard solution.*—1 mg valeric acid/ml ether.

Procedure

To 10 ml glass tube (12 mm diameter) add 1 g rye bread, 2 ml ether containing 3% (v/v) concentrated orthophosphoric acid, and 1 ml valeric acid stock solution. Homogenize mixture by disintegrating bread with glass rod during 5 min at 0°C (ice-water bath). Inject 5 μl supernatant liquid directly onto GLC column.

To determine benzoic acid in margarine, suspend 1 g margarine in 2 ml ether containing 3% orthophosphoric acid. Add 1 ml valeric acid stock solution. Centrifuge (2800× *g*) and inject 5 μl clear supernatant liquid into gas chromatograph.

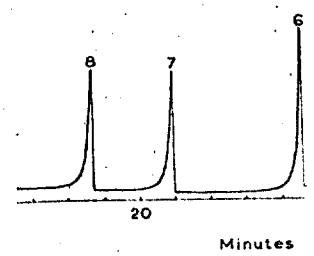


FIG. 1—Gas chromatogram of st
2, propionic; 3, isobutyric; 4,
6, valeric (internal standard);
See text for GLC c

Measure peak areas with
acids on basis of internal st
factors.

Results and Di

Figure 1 shows a typical
mixture containing 30 ng of
ular weight carboxylic acids
bowax 20M-terephthalic aci
sorbic acids as well as benz
rated on the same column.
chromatograms of low mol
ylic acids found in rye b
respectively. Valeric acid is

Because an internal stand
sary to establish the respon
free fatty acids. The respon
mined by analyzing soluti
quantities of propionic, so
leric acids (internal standa
repeated 6 times. The ave
sponse factor and the sta
summarized in Table 1. It
mine the response factors f
they depend on the colum
the carrier gas. Besides,
temperature programmin
sponse factor.

To be informed about t
ducibility of the results, t

Received February 11, 1972.

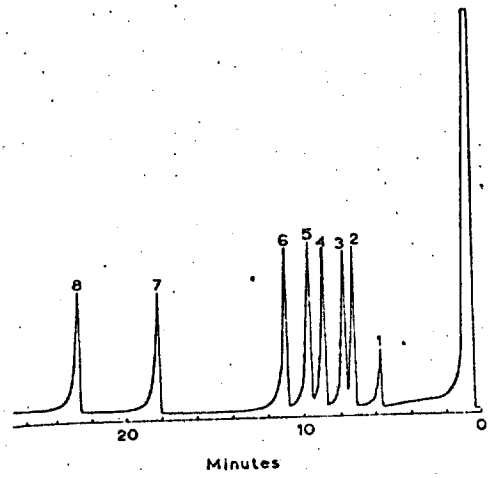


FIG. 1—Gas chromatogram of standard acids: 1, acetic; 2, propionic; 3, isobutyric; 4, butyric; 5, isovaleric; 6, valeric (internal standard); 7, sorbic; 8, benzoic. See text for GLC conditions.

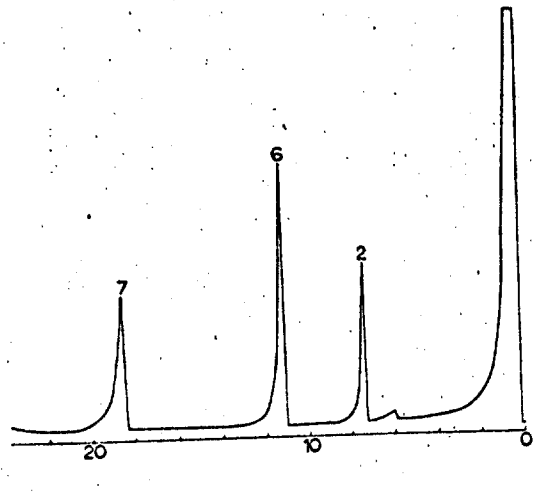


FIG. 2—Gas chromatogram of 2 preservatives found in rye bread: 2, propionic acid; 6, valeric acid (internal standard); 7, sorbic acid.

Measure peak areas with integrator; calculate acids on basis of internal standard and response factors.

Results and Discussion

Figure 1 shows a typical chromatogram of a mixture containing 30 ng of each of the low molecular weight carboxylic acids on a column of Carbowax 20M-terephthalic acid. Both propionic and sorbic acids as well as benzoic acid can be separated on the same column. Figures 2 and 3 show chromatograms of low molecular weight carboxylic acids found in rye bread and margarine, respectively. Valeric acid is the internal standard.

Because an internal standard is used, it is necessary to establish the response factor for the above free fatty acids. The response factors were determined by analyzing solutions containing equal quantities of propionic, sorbic, benzoic, and valeric acids (internal standard). The analysis was repeated 6 times. The average values of the response factor and the standard deviations are summarized in Table 1. It is essential to determine the response factors for each series, because they depend on the column and the flow rate of the carrier gas. Besides, a small change in the temperature programming may affect the response factor.

To be informed about the accuracy and reproducibility of the results, the method was checked by replicate analysis of 5 μl portions of ether extracts of a rye bread sample. The results of the test presented in Table 2 indicate that the method is accurate and precise.

The stability of the packing described above appears to be quite satisfactory. Our column was

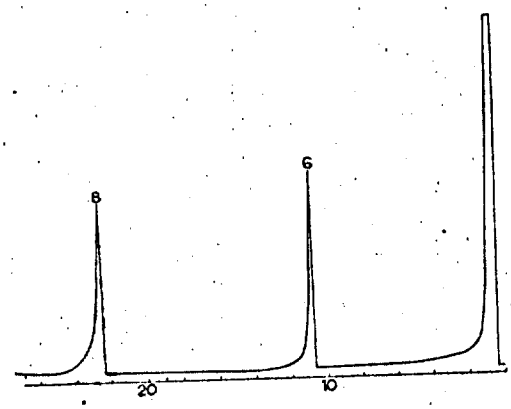


FIG. 3—Gas chromatogram of a preservative found in margarine: 6, valeric acid (internal standard); 8, benzoic acid.

Table 1. Determination of response factors for free fatty acids, calculated with reference to valeric acid

Acid	Av.	Std Dev.
Propionic	1.53	0.018
Sorbic	0.95	0.0094
Benzoic	0.88	0.0082

Received February 11, 1972.

Table 2. Determination of propionic and sorbic acids (mg/g) in rye bread and benzoic acid in margarine, 6 replicates

Acid	1	2	3	4	5	6	Av.	Std Dev.
Propionic	0.080	0.078	0.079	0.080	0.081	0.077	0.079	0.0015
Sorbic	0.151	0.143	0.161	0.148	0.152	0.141	0.150	0.0072
Benzoic	0.088	0.086	0.089	0.088	0.087	0.088	0.088	0.0010

operated continuously between 100 and 210°C for more than 4 months without deterioration.

Results obtained by the present method on 4 rye bread samples were compared with those obtained by the colorimetric method of Schmidt (6) for sorbic acid, and by the gas-liquid chromatographic method of James and Martin (7, 8) for propionic acid. The findings are given in Table 3. We obtained higher results for propionic acid with our method than with the method of James and Martin. The steam distillation involved in the latter method may have been incomplete.

The recovery of the free fatty acids was checked by adding the acids in known amounts to the rye bread sample previously analyzed. Recovery of the added free fatty acids varied from 98 to 101%.

Table 3. Comparison of present method and existing methods for determining propionic acid and sorbic acid in rye bread (4 samples analyzed)

Acid	Method	Replicates	Samples, mg/g 1	2	3	4
Propionic	GLC (7, 8)	2	0.16	0.11	0.07	0.07
	GLC	5	0.19	0.12	0.09	0.09
Sorbic	colorim. (6)	3	0.11	0.17	0.12	0.08
	GLC	5	0.10	0.17	0.12	0.09

The lowest amount of preservative detectal in foods is about 5 ng. The present method is simple and comparatively convenient means obtaining accurate quantitative results. We test the method only on rye bread and margarine. I all probability, the method may be used to est mate preservatives in other food products.

Acknowledgments

The author wishes to thank N. H. Visser fo assistance in experiments and analyses.

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By JOHN L. IVERSON (1 Washington, D.C. 20204)

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Effects of Oral Administration of Short-Chain Fatty Acids on Certain Blood and Urine Constituents of Fasted, Phlorhizin-Treated Ewes

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THE DEPENDENCE of ruminants on the end products of microbiological fermentation as sources of energy has stimulated interest in the ultimate fate of the short-chain fatty acids in such animals. A relationship may exist between the relative availability of these substances and the blood changes observed in ruminant ketosis.

Schultz and Smith[13] observed that oral administration of acetic acid only moderately increased blood ketone levels in goats, whereas a marked increase was seen after butyric, caproic, caprylic, or capric acid administration. They also observed a blood glucose-depressing effect of the latter four acids. Johnson[7] and Clark and Malan[8] reported similar effects of intravenously or orally administered butyric acid in sheep. The marked glucogenic effect of propionic acid in ruminants was reported by Schultz and Smith,[14] and its antiketogenic effect was observed by Jarrett and Potter.[6]

Phlorhizin is a naturally occurring glucoside which produces glycosuria in laboratory animals and dogs by blocking tubular reabsorption of glucose.[13] Its effects persist for several hours and, if administration is continued, a depletion of carbohydrate stores occurs, followed by hypoglycemia, ketonemia, and a marked increase in nitrogen excretion.[13]

In previous experiments,[5] it was found that fasting induced only a mild hypoglycemia and ketonemia in nonpregnant ewes. In this study, a combination of fasting and phlorhizin administration was used to render the animals hypoglycemic and ketonemic. These animals were then used to determine the glucogenic or ketogenic effects of oral administration of short-chain fatty acids.

MATERIALS AND METHODS

Adult native ewes (12), weighing between 130 and 150 lb., were allotted at random to groups of 4 animals each, and each group was used to test one of the fatty acids. Before and between fasting periods, the ewes were given approximately 2 lb. of a mixture of ground oats and corn, and also were given alfalfa hay ad libitum.

In each trial, the 4 ewes were subjected to a preliminary 48-hour period of fasting and, twice daily, to subcutaneous injections of 1 Gm. of phlorhizin dissolved in 5 ml. of ethylene glycol. At the end of this period, 2 ewes were given the fatty acid orally, while 2 control ewes were given an equal volume of water. Blood specimens were obtained at the beginning and end of the 48-hour fast and the period of phlorhizin treatment, and at one, three, six, 24, and 48 hours after administration of the fatty acid. The blood sugar levels were determined by the Somoygi-Nelson[16] method, and blood ketone levels by the method of Barnes and Wick.[1] Urine was collected with a Bardex size 12 self-retaining catheter, which was connected to a bottle containing toluene as a preservative. From 24 hours before to 48 hours after the administration of the fatty acid, urine was

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TABLE 1—Effects of Water Administration on Fasted, Phlorhizin-Treated, Nonpregnant Ewes *

Hours	Blood glucose (mg./100 cc.)	Blood ketones (mg./100 cc.)	Urinary glucose (Gm./24 hr.)	Urinary urea nitrogen (Gm./24 hr.)
−48 †	40.4	1.4	.....	.....
0 **	26.7 ± 0.7	4.5 ± 0.5	39.4 ± 2.4	13.9 ± 1.5
1	26.2 ± 1.7	.....	.....	.....
3	25.5 ± 1.2	.....	.....	.....
6	27.3 ± 1.2	4.4 ± 0.6	.....	.....
24	25.2 ± 1.5	6.9 ± 0.8	39.9 ± 2.1	13.7 ± 1.0
48	25.3 ± 1.6	10.2 ± 0.9	39.4 ± 2.3	12.9 ± 0.8

* Averages for 10 animals which served as controls for fatty acid administration trials; † fasting and phlorhizin (1 Gm. twice daily) treatments were begun; ** water was given orally.

TABLE 2—Effects of Sodium Acetate Administration on Certain Blood and Urine Constituents of Fasted, Phlorhizin-Treated Nonpregnant Ewes (Averages for 4 Animals)

Hours	Blood glucose (mg./100 cc.)	Blood ketones (mg./100 cc.)	Urinary glucose (Gm./24 hr.)	Urinary urea nitrogen (Gm./24 hr.)
−48 *	36.9	.....	.....	.....
0 †	26.6 ± 1.6	4.2 ± 0.6	45.5 ± 5.4	13.3 **
1	23.9 ± 1.2	.....	.....	.....
3	22.7 ± 1.5	.....	.....	.....
6	22.8 ± 2.8	9.0 ± 0.7 ‡	.....	.....
24	23.1 ± 3.8	9.9 ± 0.7 ‡	49.0 ± 4.9	15.7 **
48	22.8 ± 1.2	12.7 ± 2.6	48.2 ± 6.7	14.3 **

* Fasting and phlorhizin (1 Gm. twice daily) treatments were begun; † sodium acetate was given orally at a dosage of 1 grammole/100 lb. of body weight; ** 2 animals; ‡ significant difference at the 2 per cent level. Statistical significance was determined, using the Fisher "t" test, for differences in paired samples, except that blood ketone values were compared to those observed in the water-treated control animals.

collected. The 24-hour samples were pooled, and glucose and either urea or total nitrogen determinations were made. Urinary glucose levels were determined by the method of Benedict,[2] urinary urea nitrogen by the method of Folin-Youngburg,[4] and total urinary nitrogen by the Koch-McMeekin[10] method. After each trial, the animals were allowed free access to food and water for nine days; then the groups were reversed and the trial was repeated.

The fatty acids used in this study were acetic, propionic, and butyric, at dosages of 1 grammole/100 lb. of body weight. The fatty acids were neutralized with sodium hydroxide, using phenolphthalein as an indicator, and diluted to 1 liter with water before oral administration.

RESULTS

The control animals which were given water at the time of fatty acid administration showed no significant alteration in blood glucose, urinary glucose, or nitrogen levels during the subsequent 48-hour period. The average blood ketone levels increased from 4.5 to 10.2 mg./100 cc. during this period (table 1).

Acetic Acid.—The results of this trial are indicated (table 2). There was no significant change in blood glucose levels or in urinary glucose or urea nitrogen excretion after the oral administration of acetic acid. A significant increase in blood ketone levels was observed six and 24 hours after-treatment, but no change was seen 48 hours post-treatment. The significance of these observations was determined by comparison with the ketone levels of the animals after water administration (table 1).

Propionic Acid.—After propionic acid administration, there was a significant increase in blood glucose levels at the end of one, three, six, and 24 hours post-treatment; the greatest increase was seen at the end of the third hour. The levels were not significantly altered at the end of 48 hours. The blood ketone levels were significantly below those of the water-treated control animals at the end of 24 and 48 hours (table 3). These animals showed a marked increase in urinary glucose excretion during the 48-hour period after propionate administration; the average increase during the first 24 hours was 33.0 Gm., and during the second 24 hours, 18.9 Gm. Urea nitrogen excretion appeared to decrease

TABLE 3—Effects of Sodium Propionate Administration on Certain Blood and Urine Constituents of Fasted, Phlorhizin-Treated, Nonpregnant Ewes (Averages for 4 Animals)

Hours	Blood glucose (mg./100 cc.)	Blood ketones (mg./100 cc.)	Urinary glucose (Gm./24 hr.)	Urinary urea nitrogen (Gm./24 hr.)
−48 *	40.7	1.8	.....	.....
0 †	23.9 ± 2.3	4.9 ± 0.6	36.3 ± 5.3	14.4 **
1	44.4 ± 7.0 ‡	.....	.....	.....
3	59.7 ± 7.3 ‡	.....	.....	.....
6	51.8 ± 3.4 ‡	2.4 **	.....	.....
24	40.8 ± 2.1 ‡	2.9 ± 0.4 ‡	69.2 ± 6.9 ‡	11.8 **
48	28.3 ± 3.8	5.0 ± 1.5 ‡	55.2 ± 3.0	11.8 **

* Fasting and phlorhizin (1 Gm. twice daily) treatments were begun; † sodium propionate was administered orally at 1 grammole/100 lb. of body weight; ** 2 animals; ‡ significant difference at the 1 per cent level (statistical significance was determined as described for table 2).

TABLE 4—Effects of Sodium Butyrate Administration on Certain Blood and Urine Constituents of Fasted, Phlorhizin-Treated Nonpregnant Ewes (Averages for 7 Animals)

Hours	Blood glucose (mg./100 cc.)	Blood ketones (mg./100 cc.)	Urinary glucose (Gm./24 hr.)	Urinary urea nitrogen (Gm./24 hr.)*	Total urinary nitrogen (Gm./24 hr.)†
—48 **	43.8	1.1	.....	.....	.....
0 ‡	27.7 ± 1.9	5.2 ± 0.9	44.3 ± 2.3	18.0 ± 1.1	20.5 ± 2.7
1	18.7 ± 1.8 §	15.0 ± 0.3 §	.....	.....	.....
3	16.5 ± 1.6 §	24.1 ± 2.4 §	.....	.....	.....
6	19.0 ± 1.7 §	22.9 ± 2.6 §	.....	.....	.....
24	27.7	17.8 ± 2.4 §	31.1 ± 3.6 §	9.5 ± 1.0 §	18.2 ± 2.3
48	26.0	12.5 ± 3.0	35.6 ± 3.4 §	13.0 ± 1.4	15.0 ± 0.5

* Four animals; † 3 animals; ** fasting and phlorhizin (1 Gm. twice daily) treatments were begun; ‡ sodium butyrate was given at a dosage of 1 grammole/100 lb. of body weight; § statistically significant differences at the 2 per cent level, determined as described for table 2.

after propionate treatment in the 2 animals studied.

Butyric Acid.—Butyric acid administration was followed by a significant decrease in blood glucose levels at the end of one, three, or six hours post-treatment; no effect was seen at 24 or 48 hours. Blood ketone levels were markedly increased at the end of one, three, six, and 24 hours, with no change seen by the end of 48 hours when compared with the water-treated control animals (table 4). The levels increased from an average pretreatment level of 5.2 mg./100 cc. to an average high of 24.1 mg./100 cc. by the end of three hours.

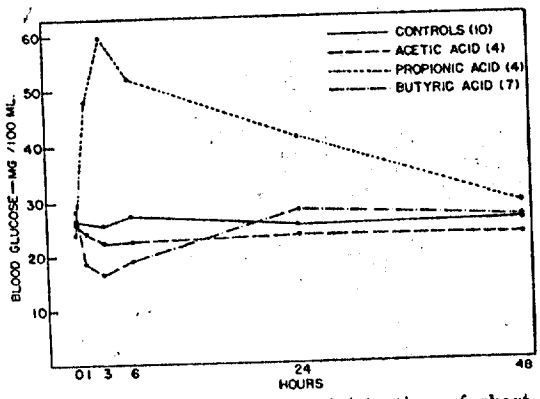
The 24-hour urinary glucose levels were decreased after butyrate treatment, as were the urinary urea nitrogen and total nitrogen levels. High 24-hour levels of urea nitrogen (18.0 Gm.) were present in the urine before the administration of butyrate. This allowed the decrease to be large without the subsequent levels being substantially below those observed in the water-treated control animals.

Discussion

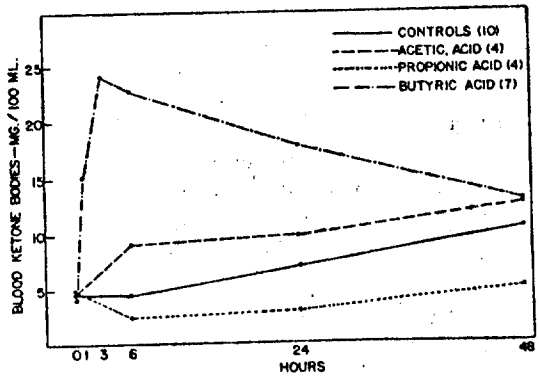
The three fatty acids studied in these trials have markedly different effects upon blood glucose and blood ketone levels. Propionic acid appears to be rapidly absorbed after oral administration, and readily converted to glucose. This conversion occurs in the liver,[9] where propionic acid is first converted to succinyl-Co A.[8]

The degree of hyperglycemia observed in these trials resembles that observed in goats by Schultz and Smith[14] and in fasted or fistulated sheep by Johnson[7] and Clark and Malan.[3] The increase in glucose excretion of approximately 52 Gm. (0.3 moles) during the 48-hour post-treatment period, when compared with an average dose of 1.4 grammole of propionic acid, indicates that over 40 per cent of the acid was recovered as urinary glucose. This calculation is based on the conversion ratio of 2 grammoles of propionic acid to 1 grammole of glucose.

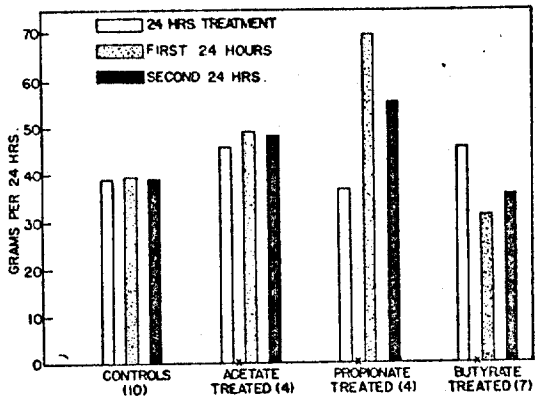
The blood glucose-depressing effect of butyrate has been observed in goats by



Graph 1—Effects of oral administration of short-chain fatty acids on blood glucose levels of phlorhizin-treated ewes fasted for 48 hours.



Graph 2—Effects of oral administration of short-chain fatty acids on blood ketone levels of phlorhizin-treated ewes fasted for 48 hours.



Graph 3—Effects of oral administration of short-chain fatty acids on urinary glucose levels of phlorhizin-treated ewes fasted for 48 hours.

Schultz and Smith,[13] and in ewes by Clark and Malan.[3] No satisfactory explanation has been given for this effect. In these trials, liver glycogen stores undoubtedly were depleted before the administration of the butyric acid, so that the depression of blood glucose levels was clearly seen. Schultz and Smith [14] observed no decrease in blood glucose after administration of ketone substances and concluded that the depression in blood glucose occurs during the conversion of butyric acid to ketone substances.

The apparent reduction in both urea nitrogen and total nitrogen excretion following butyric acid administration could be due either to a reduction in exogenous protein available or to a decrease in endogenous protein catabolism. The data suggest that the amount of exogenous nitrogen available to these animals was still high at the time of the butyrate treatment and that the addition of this material to the rumen contents interfered with microbiological synthesis of protein. Information relative to urea and total nitrogen excretion in an animal in a postabsorptive state would be desirable for comparison.

The antiketogenic effect of propionic acid which was noticed in these trials appears to be correlated with its glucogenic effect. The mild ketogenic effect of acetic acid would seem to indicate that the greatest proportion of this material is metabolized by some other route than through ketone production. Reid,[12] through arterio-venous studies of acetate levels in the heads of sheep, concluded that acetate is utilized directly by peripheral tissues in these animals.

The marked ketogenic activity of butyric acid, which was observed through the first 24 hours, is similar to the findings of Schultz and Smith [13] in goats. The rapid conversion of butyric acid to ketone bodies by the rumen epithelium which was reported by Pennington [11] would explain the rapid rise in blood ketones which was observed. In comparing the ketogenicity of butyrate with acetate, it should be stated that, in these trials, equal molar quantities were given, making the carbon content of the butyrate twice that of the acetate.

SUMMARY

The fasted, phlorhizin-treated sheep used in these trials appear to demonstrate the effects of oral administration of short-chain fatty acids more clearly and distinctly than do normal or fistulated animals.

1) When acetic acid was given, the blood ketone levels were moderately increased, but there were no changes in blood glucose or urinary urea nitrogen levels, or in the degree of glycosuria.

2) With propionic acid, blood glucose levels were markedly increased, the blood ketones were decreased, and the glycosuria greatly intensified.

3) With butyric acid, blood glucose levels were depressed, blood ketones were markedly increased, and glucose excretion was reduced. Decreases in total urinary nitrogen and urinary urea nitrogen were also observed after butyrate administration.

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J. Physiol. (1960), 154, *pp.* 115–132
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ABOMASAL SECRETION IN THE SHEEP

BY K. J. HILL

From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(*Received* 8 *May* 1960)

Anatomically and histologically the true gastric secretory stomach of the ruminant, the abomasum, is strictly comparable to the simple stomach of other mammals. The composition of the gastric juice is similar to that found in other animals and it is reasonable to assume that the basic mechanisms governing secretion are also essentially similar. The cranial part of the ruminant digestive tract is, however, considerably modified for the storage and fermentation of plant material and it is of interest to ascertain to what extent the function of the abomasum is modified by the three compartments of the forestomach which lie between it and the oesophagus. Previous investigations (Hill, 1955) have shown that the continuous flow of ingesta from the reticulo-rumen to the abomasum is responsible for its continuous secretory activity, and that the abomasal glands are not able to secrete spontaneously. The experiments to be described were carried out to obtain further information about the factors responsible for initiating and maintaining abomasal secretion.

METHODS

Surgical preparations

One- to two-year-old Clun Forest sheep were taken from pasture and placed in individual pens in the animal house, where they were allowed a week or two to become acclimatized before operation. Mineral supplements and a daily ration of 1000 g of chopped hay were provided for all animals.

Rumen fistulae were prepared in all animals and were fitted with polyvinyl cannulae (Jarrett, 1948).

Abomasal fistulae. Perspex cannulae, 16 mm diameter and fitted with screw caps, were inserted into the pyloric region of the abomasum, and brought out through the abdominal wall.

Crocker–Markowitz fistula. The duodenum was transected immediately caudal to the pyloric sphincter and a stainless-steel cannula (external diam. $\frac{7}{16}$ in. (11 mm), internal diam. $\frac{3}{16}$ in. (9·5 mm)) inserted into each end. The stems of the cannulae were brought out through the body wall and continuity of the digestive tract re-established by connecting the two cannulae with plastic tubing (Dougherty, 1955).

Vagally innervated abomasal pouches were prepared in nine sheep by a modified Hollander technique (Gregory, Hallenbeck & Code, 1942). Perspex or stainless-steel cannulae with

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stems of 16 mm diameter were placed in the pouches and brought out through stab wounds in the abdominal wall. The difficulties associated with the preparation of gastric pouches in ruminants have been discussed elsewhere (Hill & Gregory, 1951; Masson & Phillipson, 1952) but attention should again be directed to the importance of securing adequate drainage of the pouches, since the many large folds of mucosa frequently block the pouch end of the cannula and interfere with the collection of gastric juice. This difficulty was overcome in the present work by placing in the pouch a soft polyvinyl drainage tube made in the form of a perforated hollow sphere approximately 4 cm in diameter and with a stem 8 cm long. The drain was inserted into the cannula and pushed up into the pouch in order to hold the mucosal folds apart and so allow the gastric juice to drain freely. The experimental observations described here were made several months, and in some cases a year or longer, after the pouches had been made.

Although a few experiments were carried out in the animal house the sheep were usually taken to the laboratory and placed in stocks. After a time they became accustomed to this procedure and would stand quietly in the stocks for many hours. Half-hourly samples of gastric juice were collected in a graduated centrifuge tube attached to a small plastic funnel; this was held under the pouch cannula by a cord secured around the sheep's body.

Oesophagostomy. Two sheep were operated upon by the two-stage method described by Friedman (1951). In one sheep (No. 30) oesophagostomy with a 5-day interval between Stage I and Stage II was performed 3 months after an abomasal pouch had been prepared. In the other one (No. G 103) Stage I was performed before making the pouch and Stage II 3 months afterwards; both animals recovered rapidly from the second stage of the oesophagostomy and ate well for several days, the food boluses being collected as they dropped from the oesophagostomy and returned to the animal through the rumen fistula. When this operative technique is used in the dog, the sides of the oesophagostomy can be closely approximated by placing a bandage around the neck, and the animal can then eat normally. In the sheep, although the edges of the incision were brought together in a similar manner, food boluses were always ejected during eating. This may have been because the boluses, which were 8–10 cm long and 25–35 mm in diameter, were too bulky to pass through the slightly narrower oesophagus at the operation site. Saliva was lost continuously from the oesophagostomy and although this was collected and returned to the rumen the loss may have been a factor which contributed to the decline in general condition which occurred in both animals 4–5 days after operation. Both animals attempted to ruminate but this merely resulted in the loss of regurgitated rumen contents through the oesophagostomy. Sham-feeding experiments were therefore done on these animals during the first few days after the oesophageal stomata had been established.

Venous catheterization. Small-bore (external diam. 1·5 mm, internal diam. 1·0 mm) polythene tubing was introduced through a wide-bore needle into the jugular vein and held in position with sticking plaster for the duration of each experiment. Heparinized saline (10 u./ml. Liquemin; Roche Products, Ltd.) prevented clot formation in the catheter.

Analytical

pH was determined with a glass electrode (Pye Universal pH Meter, Cambridge). Free and total acid of the gastric juice was determined by electrometric titration with 0·1N-NaOH to pH 3·5 and 9·6, respectively. Peptic activity of the gastric juice was estimated by Hunt's (1948) method. Blood sugar was estimated by the Shaffer–Somogyi method (Shaffer & Somogyi, 1933). Volatile fatty acids were determined by steam distillation, with precautions to exclude CO_2, and titration of the distillate with carbonate-free 0·02N-NaOH and phenolphthalein as indicator (Annison, 1954).

RESULTS

The effect of a meal on secretion from abomasal pouches

Secretion of gastric juice was first observed under feeding conditions which simulated, as far as possible, those at pasture. The animals remained in their pens in the animal house and consecutive hourly collections of juice were made. Food was provided *ad lib.* and was eaten by the animal intermittently during the day. Under these conditions, although there was continuous secretion of acid gastric juice, there were minor fluctuations in the amount and acidity of the juice secreted each hour and an over-all trend towards higher secretory rates between 5.0 a.m. and 2.0 p.m. Over a 24-hr collection period the average hourly output of free acid was usually about 1·70 m-equiv, and of total acid about 1·90 m-equiv. Comparatively large volumes of gastric juice were secreted by the pouches; sheep H 149 for example, produced an average of 526 ml./day with a total acidity of 88·5 m-equiv/l. during a collection period of 12 days. If, as seems likely from inspection of its area, the pouch mucosa formed about 12 % of the total secretory mucosa of the abomasum, the total daily production of gastric juice would be of the order of 4·0–4·5 l.

When animals were deprived of food for 24 hr, the volume and acidity of the abomasal secretion were reduced although there were still minor fluctuations in the rate of secretion. Feeding was then followed by a marked increase in secretion and this was found to be even more pronounced in sheep which had been in the animal house for long periods; possibly because these animals ate a large proportion of their daily ration in a very short space of time. Figure 1 illustrates the secretory response obtained when 400 g of dried grass cubes were eaten during a half-hour period. The first 30-min sample showed an increase in the volume and acidity of the juice and this became maximal 1–1½ hr after the start of feeding, gradually subsiding over the next 3 hr. When chopped hay or green stuff was eaten a similar secretory response was obtained but it tended to be short-lived. Collection of juice at 10 min intervals during the feeding response showed that the onset of secretion occurred within 10 min of the start of feeding and became maximal in about 60 min. Occasionally in the feeding experiments secretion was depressed during the first 30 min after feeding. This was particularly so if the secretory rate before feeding was at a relatively high level.

Although similar increases in volume and acidity of the juice were obtained in response to feeding from all the sheep with pouches, there were individual differences in pepsin secretion. Thus in Sheep 24 (Fig. 1) the pepsin concentration remained practically unchanged during the feeding response, although the output was naturally elevated when the secretory

rate was high, maximum output being obtained during the early stages of the secretory response. Sheep H.160 and 37, on the other hand, showed an appreciable increase in the concentration of pepsin in the gastric juice and consequently a greater output of pepsin. The maximum increase in pepsin concentration occurred 1–1½ hr after the start of feeding and coincided with peak volume and acid output. In two further sheep, K94 and 30, the pepsin concentration and output although increased, were less than those observed in Sheep H.160 and 37. Atropine (10 mg subcutaneously) completely inhibited the feeding response.

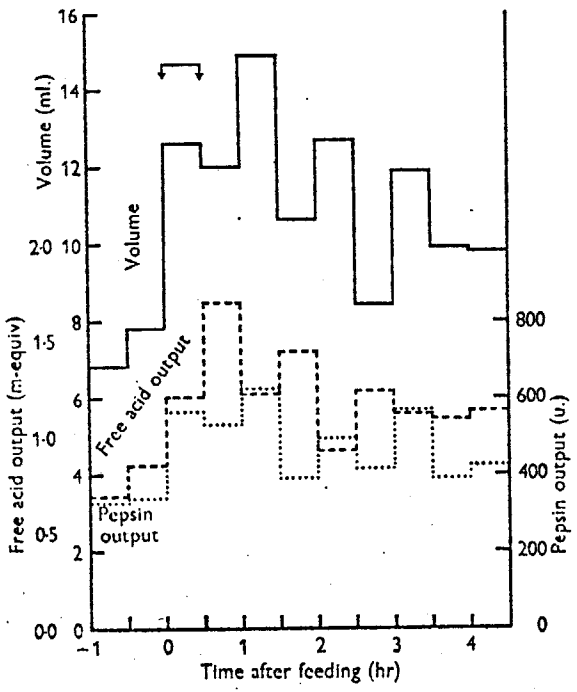

Fig. 1. The effect on abomasal secretion of eating a meal (400 g dried grass cubes, between arrows). Sheep 24. Modified Hollander pouch.

Another feature observed in animals which had become accustomed to the feeding regime was what appeared to be a conditioned secretory response to feeding. Thus, when an animal had been fasting for 24 hr and the secretory activity of the abomasum had fallen to a low level, there was sometimes a transient increase in secretion at the usual feeding time. This response was not observed when the reticulo-rumen had previously been emptied of ingesta. Feeding other sheep in the presence of the experimental subject occasionally resulted in a slight increase in abomasal secretion as did the sight and smell of food.

The effect of vagal denervation on abomasal secretion was studied in four sheep with Heidenhain pouches. In all these animals the amount of juice secreted in response to a meal was considerably less than from innervated pouches of comparable size, and it was frequently of very low acidity. The time relations of the secretory response to a meal were, however, comparable to those of an innervated pouch.

The flow of ingesta during feeding

The rate of reticulo-rumenal contractions increases when a meal is eaten and it seemed probable, therefore, that there would be a concomitant increase in the flow of ingesta into the abomasum, which might be responsible for the secretory response observed after feeding. This point was investigated in Sheep G93, which had a modified Crocker–Markowitz fistula. During experiments the connecting tubing between the two cannulae was removed and the ingesta which issued from the cranial cannula were collected and measured. They were then returned to the duodenum through the second cannula. When the animal was deprived of food the flow of ingesta from the cannula adjacent to the pylorus was greatly reduced, but eating a meal then provoked an increase in flow which was maximal during the first hour after the start of feeding. This was followed over the next 3–4 hr by a gradual reduction to the pre-feeding level (Fig. 2).

The passage of relatively large volumes of ingesta into the abomasum demonstrated in this way would presumably bring about some degree of distension of this organ and since distension of the pyloric antrum is known to stimulate gastric secretion in other animals further experiments were carried out in which the effect of pyloric distension on abomasal secretion was investigated. Animals prepared with abomasal pouches and pyloric fistulae were used and the pyloric region was distended by the inflation (40 ml. air) of a balloon passed into the abomasum through the fistula. The results were variable but in three experiments out of six secretory responses were provoked by moderate distension of the pylorus (Fig. 3). Ash (1959) has recently reported that distension of the main body of the abomasum with a balloon containing 250–500 ml. 0·9 % NaCl solution also stimulates the secretion of gastric juice.

The effect of sham-feeding on abomasal secretion

Several feeding experiments were done on Sheep 30 and Sheep G103 before oesophagostomy and in all of them typical secretory responses were obtained. After oesophagostomies had been established four 'sham-feeding' experiments were performed whilst the animals were still in good condition. In none of them was a secretory response obtained. It is well

known, however, that in the dog the 'sham-feeding' response is capricious and easily inhibited by pain or apprehension and therefore the negative evidence obtained in the sheep did not necessarily constitute very good proof of the absence of a cephalic phase of secretion. Because of the peculiar nature of the ruminant digestive tract there is, however, no real need to perform oesophagostomy in order to observe 'sham-feeding', since a form of this may be simulated by emptying the reticulo-rumen. Because of the large capacity of the reticulo-rumen, food, when eaten, then merely

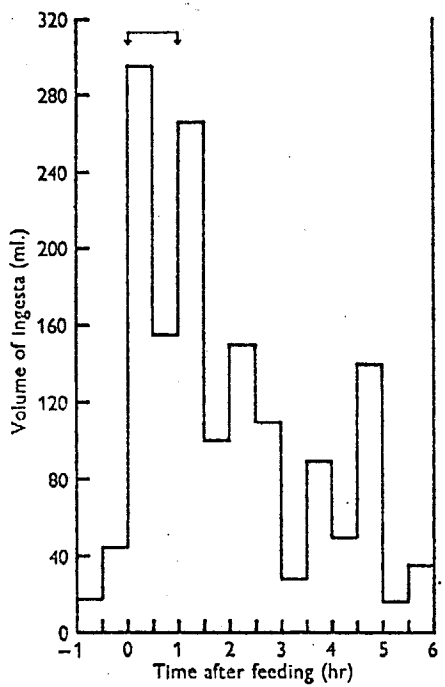

Fig. 2. The effect of eating a meal (500 g chopped hay, between arrows) on the flow of ingesta from the abomasum. Sheep G.93. Crocker–Markowitz fistula.

passes to the rumen and remains there for several hours without apparent change, providing the forestomachs have been completely emptied and washed out. Further experiments were therefore carried out in which such a preparation was used. After the reticulo-rumen had been emptied and washed free of ingesta the secretion from the abomasal pouches was much reduced in volume and contained no free acid. Food was then given to the animals and by observation through the rumen fistula it was possible to see the swallowed boluses entering the rumen where they gradually accumulated. The pouch secretion continued to be scanty and did not contain any free acid (Fig. 4). This was in marked contrast to the large secretory

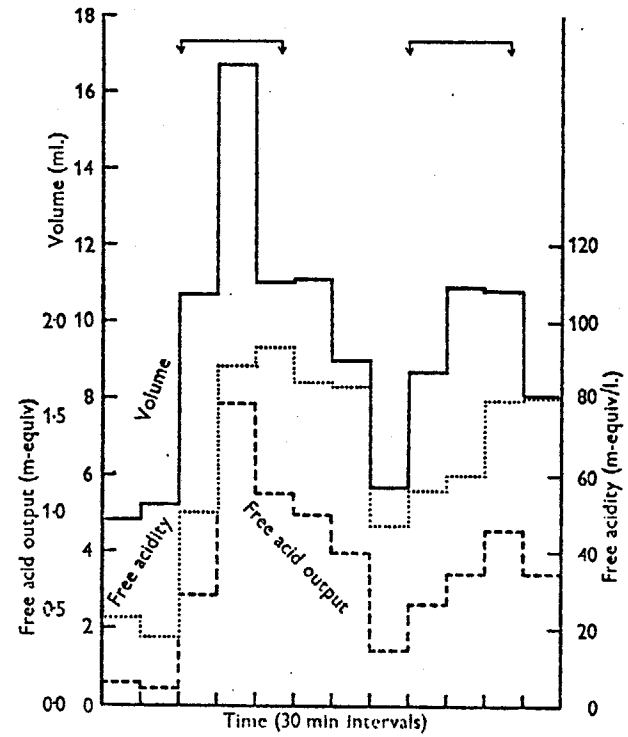

Fig. 3. The effect on abomasal secretion of distension of the pyloric region of the abomasum; between arrows, pylorus distended with balloon (40 ml. air). Sheep 42. Modified Hollander pouch.

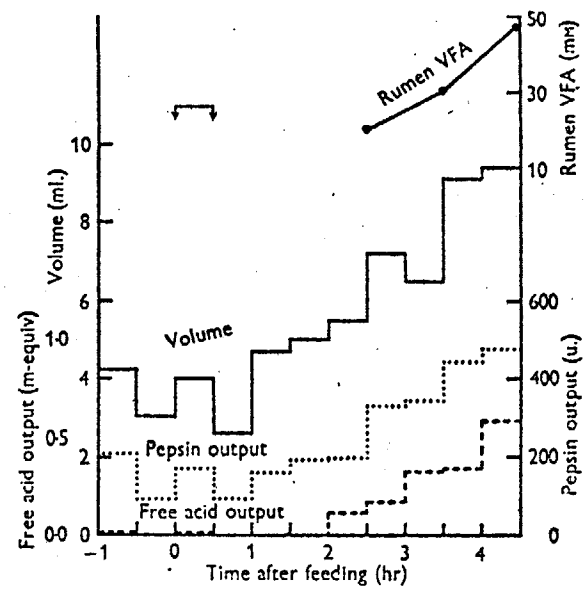

Fig. 4. The effect on abomasal secretion of eating a meal, 150 g chopped hay (between arrows); reticulo-rumen empty of ingesta. Sheep H.160. Modified

responses to feeding obtained from the same animals when the forestomachs contained ingesta. No effect was observed on pepsin secretion in any of the animals, even though two of them, H. 160 and 37, had shown increases in both concentration and output of pepsin after normal feeding. In several of these experiments there was a slight to moderate increase in secretion some 2–2½ hr after feeding. Since 'sham-feeding' and feeding with the reticulo-rumen empty failed to provoke a secretory response from the abomasum it appeared that the large increase in secretion observed after normal feeding was caused by an accelerated flow of ingesta into the abomasum. There was, however, marked stimulation of pepsin secretion in some of the feeding experiments and as pepsin secretion is generally considered to be under nervous control it seemed desirable to investigate further the possibility of vagal participation in the secretory response.

The effect of vagal stimulation on abomasal secretion

Vagal stimulation brought about by insulin hypoglycaemia has been employed extensively in studies in gastric secretion (see Bachrach, 1953) and although previous investigations (Hitchcock, Karvonen & Phillipson, 1948; Hill, 1952*b*) had indicated that insulin hypoglycaemia was inhibitory to abomasal secretion it was deemed worth while to reinvestigate this point.

Sheep H 160 and 37 were deprived of food for 24 hr and a fine polythene catheter was inserted into the jugular vein immediately before the experiment. This made it possible to give injections and to remove blood samples with minimal disturbance to the animal. After a control period insulin (Burroughs Wellcome, 40 u./ml.) was injected intravenously (0·5 u./kg). For the next 30 min there was either no change or a slight depression in secretion, but thereafter the volume and acidity increased and reached maximum values 1–2 hr later. This secretory response coincided with the fall in blood sugar and was maximal at the time of most pronounced hypoglycaemia. The pepsin concentration in the juice was slightly depressed immediately following the insulin injection and then gradually increased until it was approximately four times greater than the pre-injection level. Pepsin output was markedly elevated during the maximal volume response (Fig. 5). Although these results suggested that gastric secretory fibres were present in the vagi, another possibility was suggested by the results of previous investigations, which have shown that insulin hypoglycaemia also produces supermotility of the rumen and reticulum (Le Bars, Nitescu & Simmonet, 1953; Hill, 1954). It might therefore be that the insulin-stimulated secretion was caused by an augmented flow of ingesta to the abomasum. This point was examined in experiments in which the passage of ingesta to the abomasum was prevented by emptying the reticulo-rumen before the administration of insulin.

As in the experiments described previously the pouch secretion, after the forestomachs had been emptied, was reduced in volume and contained little or no free acid. After the intravenous injection of insulin (0·5 u./kg) secretion of juice was depressed for 30–60 min, and was then only slightly stimulated. However, the pepsin concentration of this juice was again increased to about four times the pre-injection value, although the output was a great deal less than that obtained when the reticulo-rumen contained ingesta (Fig. 6).

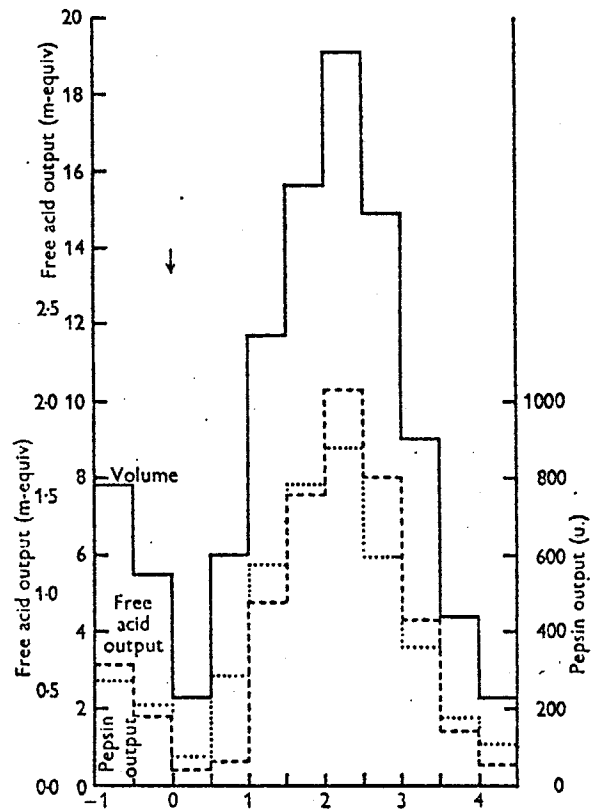

Fig. 5. The effect on abomasal secretion of insulin 0·5 u./kg I.V. at arrow. Reticulo-rumen containing ingesta. Sheep H.160. Modified Hollander pouch.

Carbamyl-choline chloride (Carbachol; British Drug Houses) given intravenously in a dose of 0·5 mg to two sheep with abomasal pouches caused a pronounced increase in the volume, acidity and pepsin concentration of the juice and in the output of acid and pepsin. The same dose of carbachol given to these sheep after the reticulo-rumen had been emptied produced comparable stimulation of pepsin secretion but a smaller acid and volume response.

Volatile fatty acids and abomasal secretion

On completion of those experiments in which the reticulo-rumen had been emptied it was the usual procedure to replace the ingesta through the rumen fistula. Whilst doing this it was noticed that there was frequently a sudden enhancement of abomasal activity. Although the obvious explanation was that this was brought about by the resumed flow of ingesta to the abomasum, it seemed possible that fermentation products present in the ingesta might have stimulated secretion after they had been absorbed from the rumen.

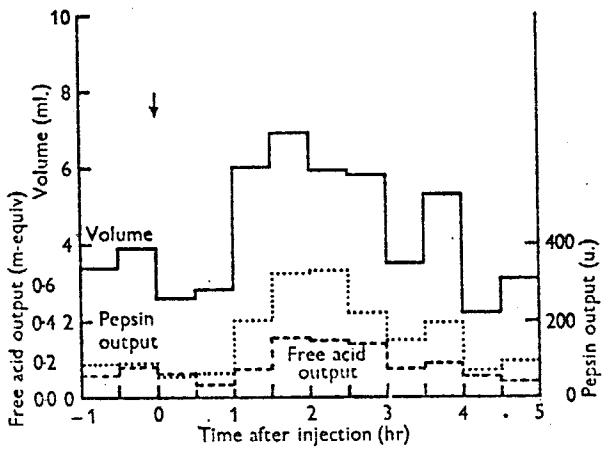

Fig. 6. The effect on abomasal secretion of insulin 0·5 u./kg i.v. at arrow. Reticulo-rumen empty of ingesta. Sheep H.160. Modified Hollander pouch.

Confirmation of this idea was obtained from certain of the experiments in which sheep were given a meal after the reticulo-rumen had been emptied. As described previously there was, in some of these experiments slight stimulation of secretion 2–2½ hr after feeding. Because of this time lag the secretion then occurring was unlikely to have been associated with the act of eating, and since the food boluses, when swallowed, entered the ventral sac of the rumen and remained there it was also unlikely that any material could have passed on to the abomasum. It was noted, however, that the food boluses lying in the ventral sac gradually lost their discrete nature and became semi-solid in consistency. This was probably partly because of their admixture with saliva but it may have been because some degree of bacterial fermentation had occurred. It is difficult to remove through a relatively small rumen fistula all traces of ingesta from the rumen and the changes observed might well have been brought about by bacteria which had remained in the rumen. As an indication, therefore, of whether or not fermentation had actually occurred in the rumen the

concentration of volatile fatty acid (VFA) was determined in the ingested food material. In the 2 hr after feeding the food boluses remained relatively solid and it was only after this time that it was possible to obtain adequate fluid samples for VFA estimation. Their concentration was then found to increase progressively. It was during this period that the increase in secretion from the abomasal pouch occurred (Fig. 4).

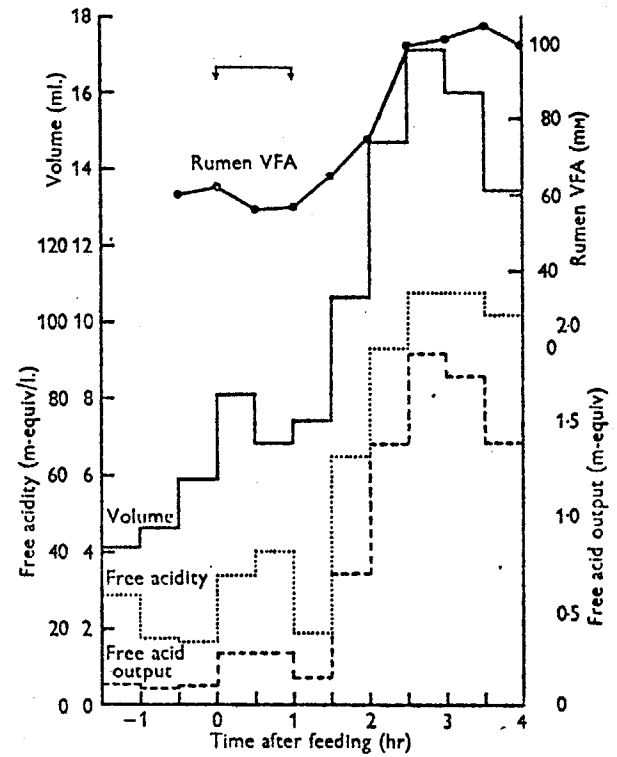

Fig. 7. The effect of eating a meal (between arrows) on abomasal secretion and on the concentration of volatile fatty acid (VFA) in the rumen: the meal was 380 g chopped hay + 200 g oats. Sheep K94. Modified Hollander pouch.

Because of this apparent correlation between the increase in concentration of rumen VFA and the increase in abomasal secretory activity in sheep with the rumen empty the rumen VFA and abomasal secretory patterns were compared in normal feeding experiments (Fig. 7). In most of these experiments in which hay and oats were fed it was usual for the rumen VFAs to increase rapidly to between 100 and 120 m-mole/l. during the 2–3 hr after feeding and then to decline slowly over the subsequent few hours. For the first 1–2 hr after feeding there was always good correlation between the rapid increase in VFAs and the increase in secretion, but after this time there was only rarely any correlation between the two. It was in fact usual for the secretory response to subside about this time

while the VFA remained high or declined only slightly. Neither does there appear to be any relation between the actual concentration of VFA in the reticulo-rumen and the rate of secretion from the abomasum, since considerable secretion occurred after kale had been eaten when the rumen VFA remained relatively low.

In an attempt to separate the secretory effects of an increased flow of ingesta to the abomasum caused by eating from possible stimulatory effects of VFAs, food was placed directly in the rumen through the rumen fistula. Five such experiments were carried out and in each of them there was a small to moderate secretory response. VFA production usually increased more slowly than after normal feeding and maximum concentration was not attained until some 3–4 hr after food had been placed in the rumen. Abomasal secretion followed a similar time course.

These experiments indicated that fermentation products, possibly fatty acids, might act as stimulants to gastric secretion, and consequently the effect of adding individual fatty acids to the rumen contents was investigated. Figure 8 shows an experiment in which three separate doses of 250 m-mole of acetic acid neutralized to pH 5·5 with NaOH were added to the rumen contents at hourly intervals. The animal was deprived of food for 24 hr before the experiment and during the control collection period the secretion was much reduced in volume and acidity whilst the VFA concentration in the rumen remained steady at a relatively low level. During the 30 min following the addition of the first dose of acetic acid there was considerable stimulation of secretion and this was even greater after the addition of the two remaining doses. The level of total VFA in the rumen increased rapidly to a maximum of 105 mM, which was well within the normal range. One hour after the addition of the last dose of acetic acid there was marked reduction in the secretion even though the VFA concentration in the rumen had not declined. Pepsin concentration and output were increased to approximately twice the control level. Acetic acid adjusted to pH 6·0, 6·2 and 6·5 with NaOH gave similar results as did the addition of acetic acid to the empty rumen.

Experiments were also done in which similar amounts of propionic acid and butyric acid were added to the rumen. Both of them were found to stimulate abomasal secretion although to a lesser extent than acetic acid. The results with butyric acid were particularly variable, since either good or poor responses were obtained with the same dose of acid.

As it is generally held (Barcroft, McAnally & Phillipson, 1944) that the VFA produced during fermentation is absorbed from the forestomachs and that only small amounts reach the abomasum, it seemed possible that the secretagogue effects of the fatty acids were mediated after they had been absorbed and were not caused by direct stimulation of the gastric

glands after they had passed to the abomasum. Investigations on the VFA content of the abomasal ingesta after the addition of 250 m-mole of acetic acid to the rumen gave support to this view, since this did not result in any significant change in the amount of fatty acid in the abomasum. When, however, larger amounts of acetic acid were added to the rumen there was a marked rise in the VFA concentration in the abomasal ingesta.

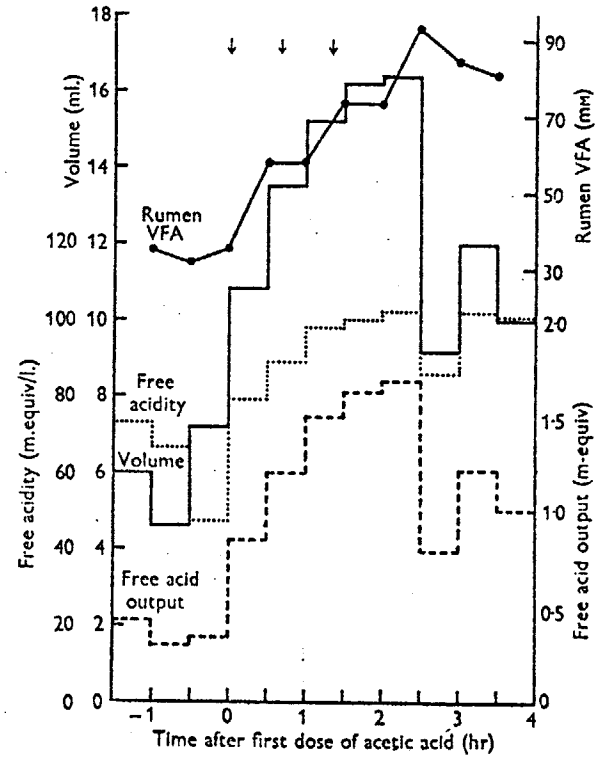

Fig. 8. The effect of adding acetic acid to the rumen contents on abomasal secretion: each arrow marks addition of 250 m-mole acetic acid, pH 5·5. Sheep H.160. Modified Hollander pouch.

DISCUSSION

Because of the relatively poor food value of plant material ruminants at pasture spend a considerable proportion of each 24 hr eating, and there is therefore frequent addition of fresh food material to the reticulo-rumen where it is subjected to microbial fermentation and whence there is a more or less uninterrupted flow of ingesta through the omasum into the abomasum. It was therefore not surprising to find continuous secretion of highly acid juice in the experiments where natural grazing conditions were simulated by frequent feeding. Under these conditions there was no obvious correlation between feeding and the secretory activity of the abomasum,

findings which are in agreement with those of Masson & Phillipson (1952). The estimated total daily output of gastric juice under these conditions was also in reasonably close agreement with the figures of these authors. Previous investigations (Grosser, 1905; Belgowski, 1912; Hill, 1952*a*, 1959*a*; Kuimov, 1952), also confirmed here, have shown, however, that when food is withheld the level of secretion is reduced and that feeding is then followed by a marked secretory response. Kuimov also observed differences in the volume of juice secreted after different foodstuffs were fed, and this finding has received some confirmation in the present investigations, where the secretory response after hay was less than after concentrate had been eaten. Variations in the gastric secretory response to different foodstuffs have been recognized in monogastric animals for many years, but they are of particular interest in ruminants where ingested food material does not pass directly to the secretory stomach and where the composition of the material which passes to the abomasum is probably of relatively constant composition. In view of the secretogogue effects of fatty acids it may be that differences in the amounts and relative proportions of these substances when concentrates are eaten are responsible for their more prolonged stimulation of abomasal secretion. Long-term experiments with animals on specific diets are needed before the influence of particular foods on abomasal secretion can be fully assessed.

The cephalic phase of gastric secretion in ruminants is generally considered to be of little significance. Thus Popov (1932), who appears to have been the first to suggest that the reflex phase of secretion is not so marked in ruminants, found little difference in the secretory behaviour of vagally innervated and vagally denervated gastric pouches in sheep. Espe & Cannon (1937), who performed oesophagostomies in calves with Pavlov pouches and carried out 'sham-feeding' experiments with milk, also concluded that the psychic or cephalic phase was absent or of little importance.

The 'sham-feeding' experiments reported here also indicate that a cephalic phase of secretion is absent in the sheep and this was confirmed by the fact that the secretory response obtained when normal animals were fed was absent when the forestomachs were devoid of ingesta. These results show that the presence of ingesta in the reticulo-rumen is necessary for the development of a secretory response. Because of the considerable increase in reticulo-rumenal movements during eating and the concomitant increase in the flow of ingesta, it seems likely that this accelerated flow stimulates the pyloric phase of secretion and is mainly responsible for the secretory response. Some supporting evidence for this is provided by the increase in secretion observed after pyloric distension. It is recognized that there are species differences in the relative importance of the different

phases of gastric secretion (James, 1957) and it is perhaps not unreasonable to assume, because of the continuous nature of digestion in the sheep, that there is no necessity for a rapid outpouring of gastric juice of high enzyme content such as is produced during the cephalic phase of secretion in the dog.

The apparent conditioned response observed in some animals at the normal feeding time may be explained by the finding of Quin & Van der Wath (1938) and Dedashev (1959) that the movements of the reticulo-rumen can become conditioned to feeding and that there is supermotility of both chambers at the expectation of food. As the response was not observed when the forestomachs were empty of food it could not have been the result of a conditioned reflex affecting the gastric glands directly. The occasional responses obtained when animals witnessed the feeding of others probably have the same explanation.

The demonstration of a secretory response to insulin hypoglycaemia was at variance with previous results obtained in the lamb (Hitchcock *et al.* 1948) and in the goat (Hill, 1952*b*), where inhibition of secretion was noted. The reason for this discrepancy is not known but the over-secretion which occurred in the present experiments when the reticulo-rumen contained food material indicated that the vagi exert considerable control over the gastric glands. That this control was, however, mainly indirect, was shown by the small response obtained when insulin was given to animals in which the forestomachs were empty of ingesta. These results again indicate that an increased flow of ingesta into the abomasum, provoked by reticular-rumenal supermotility, is the most important factor in the stimulation of abomasal secretion.

The persistence of a small secretory response to insulin hypoglycaemia after the forestomachs had been emptied did, however, show that some secretory fibres were present in the vagi. The lack of response to sham-feeding with the rumen empty therefore suggests that, if there is a vagal component in the normal secretory response to feeding, then it must be mediated through afferents so situated that they are not stimulated by sham-feeding. Such would be the case if receptors were located elsewhere than the buccal cavity and in this respect it may be pertinent to note that sensory nerve endings have, in fact, been demonstrated in the reticulo-rumen epithelium (Hill, 1959*b*). Whether these are concerned in the reflex control of gastric secretion remains to be seen but it is of interest that vagal afferents concerned with salivary secretion have already been described in the reticulo-rumen (Kay, 1958). Harper, Kidd & Scratcherd (1959) have also shown that stimulation of the central end of the abdominal vagus in the cat causes secretion of acid and pepsin in the gastric juice.

The possibility that a humoral mechanism stimulates pepsin secretion

during the pyloric phase of secretion must also be considered in view of the long time lag, observed in the feeding experiments, before maximal secretion occurred. Although there is no conclusive evidence that pepsin secretion during the pyloric phase is controlled by other than nervous means yet the demonstration of a pepsin-stimulating hormone, gastrozymin (Blair, Harper & Lake, 1953) would, if confirmed, provide an alternative mechanism for pepsin secretion in the sheep.

Whether or not vagal stimulation plays a part in the secretory process the presence of vagal innervation appears to be important in ensuring maximum responsiveness of the gastric glands. This was shown by the diminished response of denervated pouches and conforms with the general conclusion in the literature that denervation reduces the secretory activity of the secretory glands to chemical stimuli.

The finding that volatile fatty acids, particularly acetic acid, had a pronounced effect on abomasal secretion was of interest because of the large amounts of these substances produced during rumen fermentation. Since it is well established that absorption of these acids takes place almost entirely from the reticulo-rumen and omasum (Barcroft *et al.* 1944) it is possible that their secretogogue effects are produced after absorption. On the other hand Popov (1932) and more recently Ash (1959) have shown that the addition of acetic acid directly to the abomasum stimulates gastric secretion and Russev (1959) has obtained similar results with propionic acid. Ash has also shown that rumen contents, when perfused through the abomasum, will increase secretion, and the possibility therefore exists that the VFAs only exert their effect when they reach the abomasum. Since the VFA levels in the abomasal ingesta are usually very low, stimulation of gastric secretion by this means may not be of very great significance. The lack of correlation between rumen VFA and abomasal secretion in the later stages of experiments, where there had been an initial correspondence between the two, calls for further enquiry but the decline in secretion may well have been due to inhibition brought about by the presence of highly acid gastric juice in the abomasum and duodenum.

SUMMARY

1. When sheep with innervated abomasal (gastric) pouches were allowed food *ad lib.*, secretion of gastric juice was continuous and showed no obvious relation to feeding. Fasting greatly reduced secretion and the ingestion of food was then followed by a secretory response in which maximum output of acid and pepsin occurred 1–1½ hr after feeding.

2. Smaller secretory responses with similar time courses were obtained from denervated abomasal pouches.

3. In a sheep with a Crocker–Markowitz cannula feeding after the animal had been deprived of food resulted in an immediate increase in the flow of abomasal ingesta, which was maximal 1–1½ hr after feeding.

4. Distension of the pyloric region of the abomasum stimulated gastric secretion.

5. Sham-feeding and feeding of sheep in which the forestomachs were free from ingesta failed to stimulate abomasal secretion.

6. Insulin-induced hypoglycaemia produced a secretory response but this was mainly attributable to an increase in the rate of passage of ingesta to the abomasum brought about by reticulo-rumenal supermotility. Direct vagal effects on the gastric glands observed when the forestomachs were free from ingesta were confined mainly to stimulation of pepsin secretion.

7. Carbachol stimulated abomasal secretion which was not abolished by emptying the forestomachs.

8. The secretory response to a meal paralleled, in its early stages, the increase in concentration of volatile fatty acids in the rumen. Addition of food directly to the rumen also produced a secretory response, which paralleled the delayed increase in the concentration of volatile fatty acid observed under these conditions.

9. Acetic acid partially neutralized with NaOH was a potent stimulus to gastric secretion when added to the rumen. Propionic and butyric acids had smaller and less predictable effects.

10. It is concluded that the most important stimulus of gastric secretion in the sheep is the frequent passage of ingesta from the reticulo-rumen to the abomasum and that volatile fatty acids, particularly acetic acid, produced during rumen fermentation, may also play a part in the secretory response to feeding.

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POTASSIUM IN DIFFERENT LAYERS OF ISOLATED DIAPHRAGM

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(*Received* 30 *May* 1960)

Many measurements have been reported of the exchange of potassium between isolated tissues and the surrounding fluid. If the tissue is thick, then calculations lead to the conclusion that the exchange of potassium as measured in the tissue as a whole may be slower than the exchange for the individual fibres, owing to the retarding effect of intercellular diffusion (Harris & Burn, 1949; Keynes, 1954). When outward movement of ^{42}K is measured by placing the loaded muscle in flowing inactive saline, only the outer layer of cells is in an environment with negligible ^{42}K; most of the fibres are surrounded by interstitial fluid containing appreciable ^{42}K and the concentration gradient is correspondingly reduced.

There has been little experimental study of the effect of thickness on the exchange rate of tissues, and there is some evidence against the concept. Thus McLennan (1955) found only small differences at 20° C in the exchange for two muscles which were 3·5 and 0·5 mm thick, and concluded that intercellular diffusion was not in this case a rate-limiting factor. A different model has been proposed by Harris (1957) who considers that the exchange may be limited by intracellular diffusion.

If intercellular diffusion markedly affects the measured exchange rates, then it is important to establish this experimentally, so that corrections may be applied where necessary. One consequence of diffusion delays of this nature is that exchange will be non-uniform, the outer layers exchanging faster than the inner layers. It was thought possible that this might be demonstrated by slicing with a microtome.

Prolonged hypoxia produces depolarization in the deeper parts of an isolated diaphragm, while the superficial fibres show little change (Creese, Scholes & Whalen, 1958). There is also a loss of potassium, and it might be expected that hypoxia would lead to a depletion of potassium particularly from the deep tissues. This was also tested by slicing with a microtome.

Thick muscles (650 μ) have been employed, at 38° C. In these circum-

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THE EFFECTS OF THE ADMINISTRATION OF ACETIC, PROPIONIC, AND *n*-BUTYRIC ACIDS UPON THE BLOOD GLUCOSE AND KETONE BODY LEVELS OF GOATS

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Received 8 Jan 1955

WITHIN the last decade it has become increasingly evident that virtually all the carbohydrate ingested by the ruminant is degraded by anaerobic microorganisms to the volatile organic acids—acetic acid, propionic acid, and *n*-butyric acid—and that ruminants are dependent primarily upon these acids for their supply of energy.[10,11] The degree of the relative contribution of the three acids to energy production has, however, been the subject of considerable controversy.[10,11]

Apparently contradictory results, obtained by various methods of investigation used in the study of this difficult problem, have finally been largely resolved by the efforts of Phillipson and his coworkers. In their latest paper[19] they report mean absorption rates for sheep of 32 milliequivalents per hour of acetic acid, 18 milliequivalents per hour of propionic acid, and 23 milliequivalents per hour of *n*-butyric acid. They calculate that of the total energy of 25.3 kilocalories per hour represented by these amounts of acids, acetic acid accounts for 6.7 kilocalories, propionic acid 6.6 kilocalories, and *n*-butyric acid 12.0 kilocalories.

Since the advent of the use of isotopic tracers, the major pathways by which metabolites ultimately converge in the common energy-generating device known as Krebs' citric acid

cycle have been reasonably well defined in the usual laboratory animals.[11,15,16] Limited tracer studies with ruminants have yielded results consistent with the same pathways.[3,4,12,13,14]

Although these pathways indicate that propionic acid is the only one of the three acids giving rise to significant quantities of glucose, and that acetic acid and *n*-butyric acid are precursors of ketone bodies, numerous reports at variance with this conception have appeared in the literature. In view of the major energy contribution made by these acids and the obvious relationship of their metabolism to the problem of ketosis, a study was undertaken to determine whether the administration of the individual volatile fatty acids, and combinations thereof, causes changes in the blood glucose and ketone body levels consistent with the generally accepted mechanisms of metabolism. This study is the subject of this report.

EXPERIMENTAL PROCEDURE

Figures 1 to 10 summarize the experimental data. Grade female goats were employed. For the oral administration a constant-rate pump was used, the solution passing into the rumen via a tube passed through a nostril. Intravenous administration was made with a constantly supervised drip device through a small plastic tube inserted into the jugular vein.

Blood samples were collected from the jugular vein into test tubes containing sodium fluoride. Plasma glucose was determined by the method of Somogyi[27] using Benedict's[2] chromogenic reagent; the total plasma ketone bodies were determined by the method of Behre[1] modified for use with a Coleman spectrophotometer. As it was not possible to maintain a normal acid-base balance in these experiments, data on the CO_2 capacity, determined by the method of Van Slyke,[7] are included in order to indicate the degree of departure from normal conditions.

RESULTS AND DISCUSSION

CHANGES IN BLOOD KETONE BODY LEVEL

A. Effect of n-butyric acid. According to presently held concepts of metabolism, *n*-butyric acid may be used for the synthesis of fat and substances such as cholesterol, may be partially oxidized to acetoacetate and its derivatives, or may be oxidized via the citric acid cycle.[11] It thus tends to be ketogenic. That this is the case in ruminants has been shown by numerous investigators.[9,17,18,25,26] The experiments summarized in figures 1, 4, 5, 6, 7, and 8 likewise confirm this fact.

B. Effect of acetic acid. Acetic acid, in the usual experimental animals, may be used for the synthesis of fat and other compounds, may condense with itself to form acteoacetic acid and its derivatives, or may be oxidized via the citric acid cycle.[11] The question whether it is actually ketogenic or not in ruminants has been the subject of some disagreement. Schultz, Smith, and Lardy[26] in 1949, after administering acetic acid orally to goats, found no increase in blood ketones. In 1951 Schultz and Smith[25] concluded that a large excess of acetic acid causes a slight ketosis. Pennington[17] has observed a slight increase in ketone bodies in the venous blood from the rumen after acetate was placed in the rumen. On the other hand, investigators administering the acid intravenously as its sodium salt have concluded that it is ketogenic.[8,9,22,28]

Figure 3 indicates that orally administered acetic acid has a slight ketogenic effect, although only those experiments which show a drop in ketone bodies after the cessation of administration of acid rule out the possibility that the increase is due to starvation. The data of figure 9 show little ketogenic effect from orally administered acetic acid, while those of figure 10 indicate an increase in ketone bodies. When we consider that two moles of acetate must combine to form one mole of acetoacetate and that the rate of absorption of acetate is less than that of *n*-butyrate, the difference observed between the ketogenic action of the two acids is not surprising. If acetic acid is administered by both the oral and intravenous routes, as was done in the experiments summarized in figures 9 and 10, the ketogenic effect of acetic acid is apparent ($P<0.001$).*

*Probability was calculated by use of the *t* test.[6]

$\frac{F-D}{F}$'s of fig. 9 + $\frac{C-A}{C}$'s of fig. 10, $t = 9.83$, $n-1 = 5$.

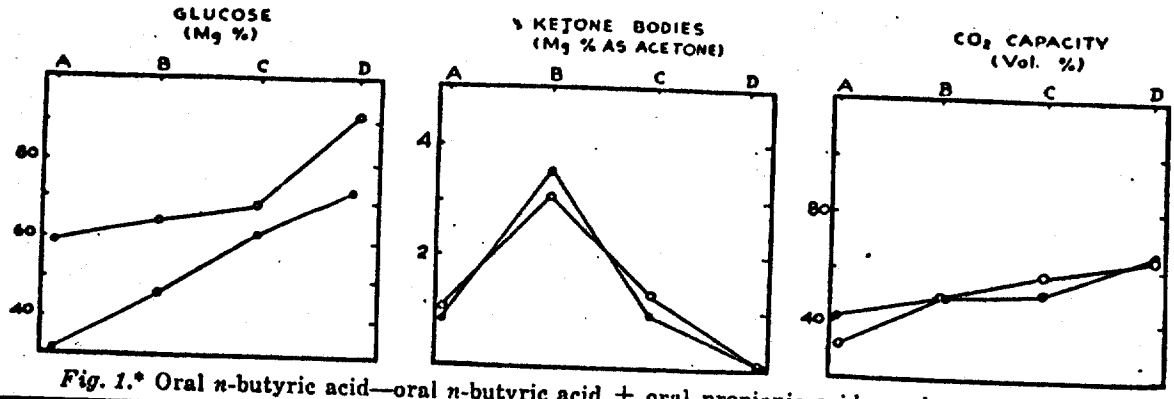

*Fig. 1.** Oral *n*-butyric acid—oral *n*-butyric acid + oral propionic acid—oral propionic acid

Hours of administration

53	24	24	24
A	B	C	D
<—Starved—>	<—0.5 mole *n*-butyric acid, 3.5 g KOH, 0.5 g NaOH/L (oral)—>	<—0.5 mole *n*-butyric acid, 0.25 mole propionic acid, 3.5 g KOH, 0.5 g NaOH/L (oral)—>	<—0.25 mole propionic acid, 1.75 g KOH, 0.25 g NaOH/L (oral)—>

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration: A-B $\overline{B_o}$	B-C B_o	B-C P_o	C-D $\overline{P_o}$
○ 1	381	25	D	1.52	1.55	0.78	0.78
● 2	382	28	D	1.30	1.30	0.65	0.59

* The following abbreviations are used in the data accompanying figures:

Hours of administration — Capital letters indicate times at which blood samples were taken and correspond to the letters on the graphs.

Stage of lactation or pregnancy — D = dry and not more than 10 weeks pregnant, ( ) = weeks of lactation, [ ] = weeks before parturition.

Rate of administration — B, P, A, G, Na = administration of *n*-butyric acid, propionic acid, acetic acid, glucose, and $NaHCO_3$ respectively in millimoles per kilogram per hour. A subscript "o" indicates that administration was oral; a subscript "v" indicates intravenous administration.

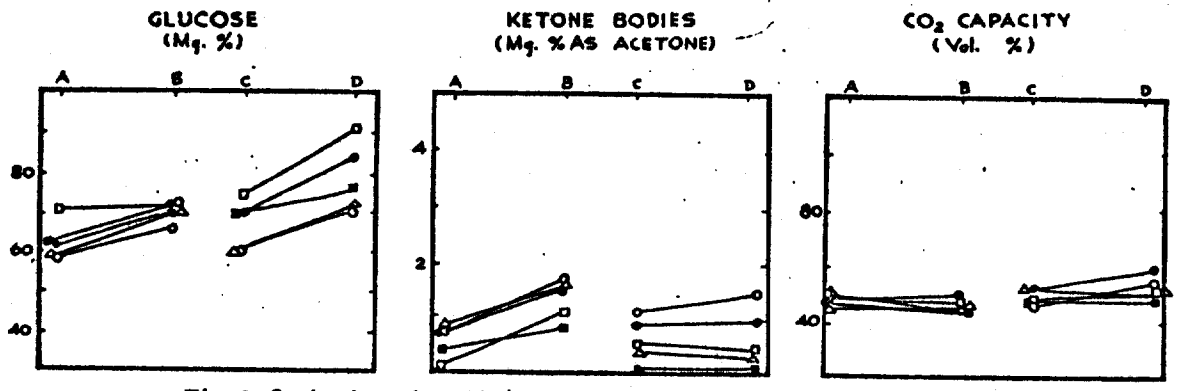

Fig. 2. Oral *n*-butyric acid + oral propionic acid — oral propionic acid

Hours of administration

55	23	25	23
A	B	C	D
<—Starved—>	<—0.5 mole *n*-butyric acid, 0.25 mole propionic acid, 3.5 g KOH, 0.5 g NaOH/L (oral)—>	<—Starved—>	<—0.25 mole propionic acid, 3.5 g KOH, 0.5 g NaOH/L (oral)—>

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration: A-B B_o	A-B P_o	C-D P_o
○ 1	347	31	D	1.27	0.69	0.69
● 2	385	24	D	1.67	0.89	0.81
□ 3	316	38	D	0.87	0.44	0.43
■ 4	377	57	D	0.43	0.22	0.22
△ 5	378	59	[10]	0.44	0.22	0.22

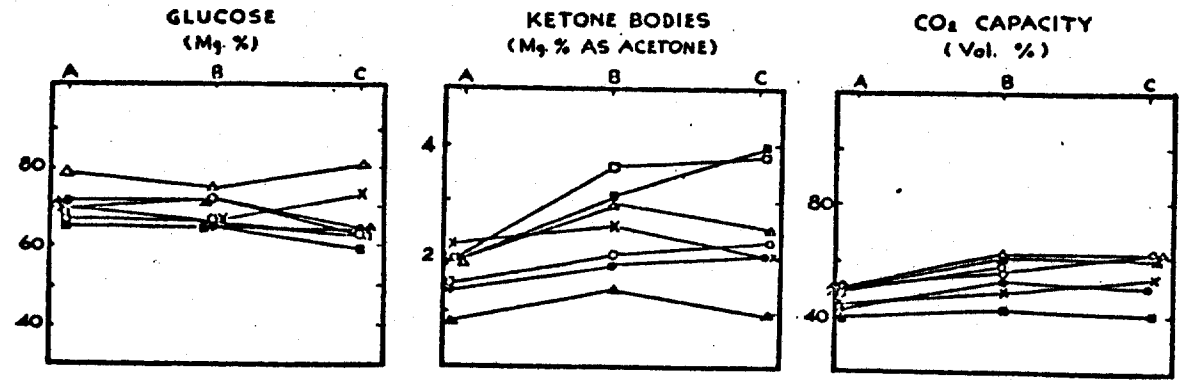

Fig. 3. Oral acetic acid

Hours of administration

53 — 24 — 24

A — B — C

<—Starved—><—0.67 mole acetic acid, 5.34 g KOH, 0.67 g NaOH/L (oral)—><—Starved—>

	Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration $\frac{A\text{-}B}{A_e}$
○	1	438	59	D	0.48
●	2	377	57	D	1.17
□	3	441	48	D	1.41
■	4	440	42	D	1.42
△	5	442	30	D	1.93
▲	6	443	39	D	1.50
X	7	385	34	D	1.61

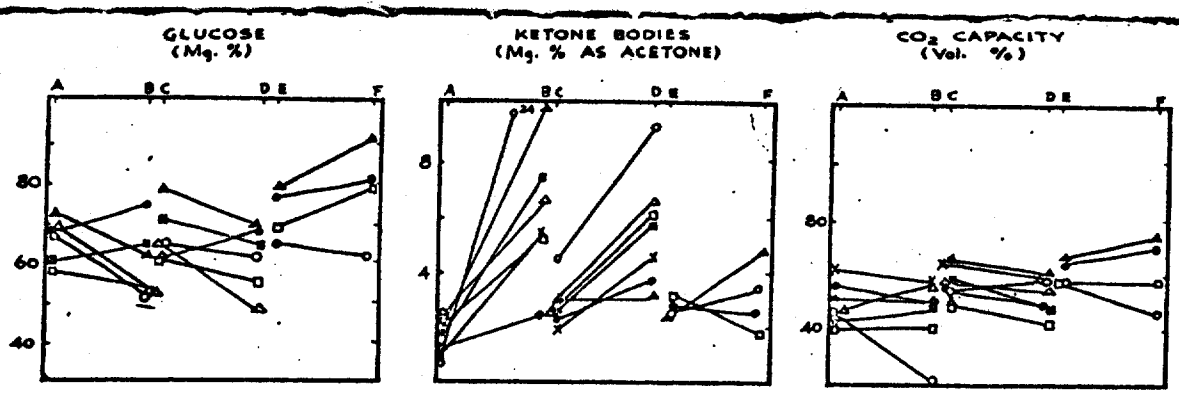

Fig. 4. Oral acetic acid + oral *n*-butyric acid—oral *n*-butyric acid—oral acetic acid

Hours of administration

53 — 24 — 24 — 24 — 24 — 24

A — B — C — D — E — F

<—Starved—><—0.67 mole acetic acid, 0.33 mole *n*-butyric acid, 8 g KOH, 1 g NaOH/L (oral)—><—Starved—><—0.33 mole *n*-butyric acid, 2.67 g KOH, 0.33 g NaOH/L (oral)—><—Starved—><—0.67 mole acetic acid, 5.34 g KOH, 0.67 g NaOH/L (oral)—>

	Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B $\frac{A_e}{}$	A-B $\frac{B_e}{}$	C-D $\frac{B_e}{}$	E-F $\frac{A_e}{}$
○	1*	377	57	D	1.96	0.98	0.99	1.96
●	2*	438	59	D	0.68	0.34	0.34	0.68
□	3*	363	41	D	1.57	0.79	0.79	1.57
■	4	440	42	D	1.31	0.66	0.66	
△	5	441	48	D	1.25	0.63	0.63	
▲	6	442	30	D	1.92	0.96	0.96	2.80
X	7	443	39	D	1.95	0.98	0.99	

* During these runs, in which no base was added to the acid, the goats developed a very severe diarrhea.

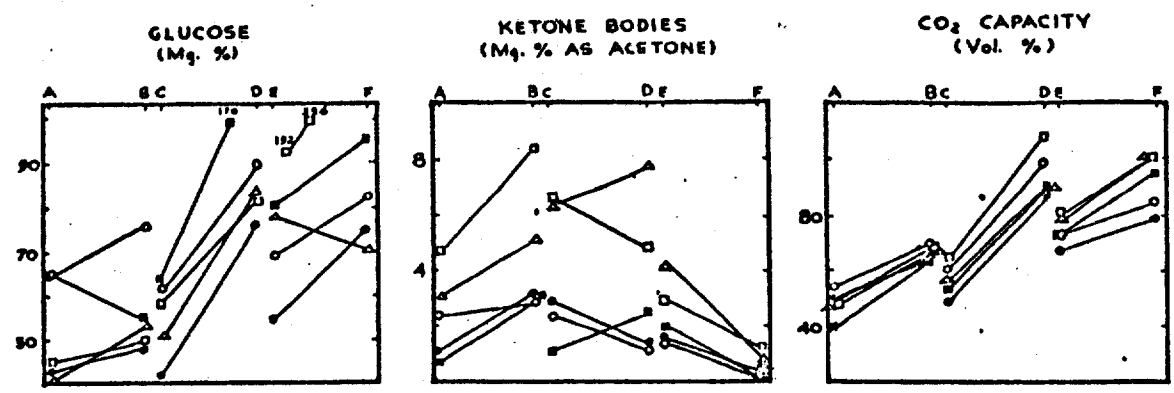

Fig. 5. Intravenous *n*-butyric acid—intravenous *n*-butyric acid + intravenous propionic acid—intravenous propionic acid

Hours of administration

24	6	18	6	18	6
A	B	C	D	E	F
<—Starved—>	<—0.375 mole *n*-butyric acid/L adjusted to pH 6.0 with NaOH (IV)—>	<—Starved—>	<—0.375 mole *n*-butyric acid, 0.375 mole propionic acid/L adjusted to pH 6.0 with NaOH (IV)—>	<—Starved—>	<—0.375 mole propionic acid/L adjusted to pH 6.0 with NaOH (IV)—>

Run	Goat no.	Weight kg	Stage of lactation or pregnancy	Rate of administration A-B Bv	C-D Bv	C-D Pv	E-F Pv
○ 1	344	30	(1)	2.36	2.50	2.50	2.50
● 2	142	63	[5]	1.19	1.19	1.19	1.19
□ 3	220	40	(2)	1.87	1.87	1.87	1.87
■ 4	316	37	(6)	2.03	2.03	2.03	2.03
△ 5	347	31	(21)	1.61	1.61	1.61	1.61

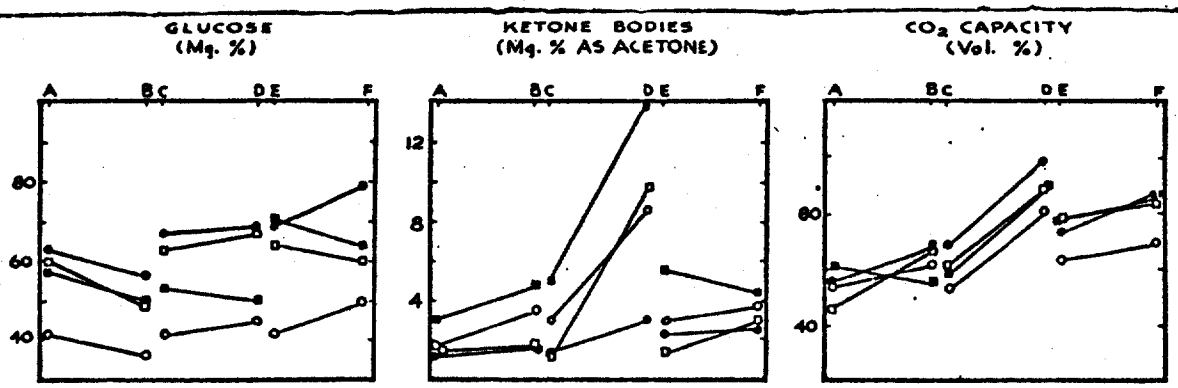

Fig. 6. Intravenous *n*-butyric acid—intravenous *n*-butyric acid + intravenous acetic acid—intravenous acetic acid

Hours of administration

24	6	18	6	18	6
A	B	C	D	E	F
<—Starved—>	<—0.375 mole *n*-butyric acid/L adjusted to pH 6.0 with NaOH (IV)—>	<—Starved—>	<—0.375 mole *n*-butyric acid, 0.375 mole acetic acid/L adjusted to pH 6.0 with NaOH (IV)—>	<—Starved—>	<—0.375 mole acetic acid/L adjusted to pH 6.0 with NaOH (IV)—>

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B Bv	C-D Bv	C-D Av	E-F Av
○ 1	142	63	[1]	1.19	1.19	1.19	1.19
● 2	344	30	D	2.50	2.50	2.50	2.50
□ 3	316	37	(6)	2.03	2.03	2.03	2.03
■ 4	347	31	(14)	1.61	1.61	1.61	1.61

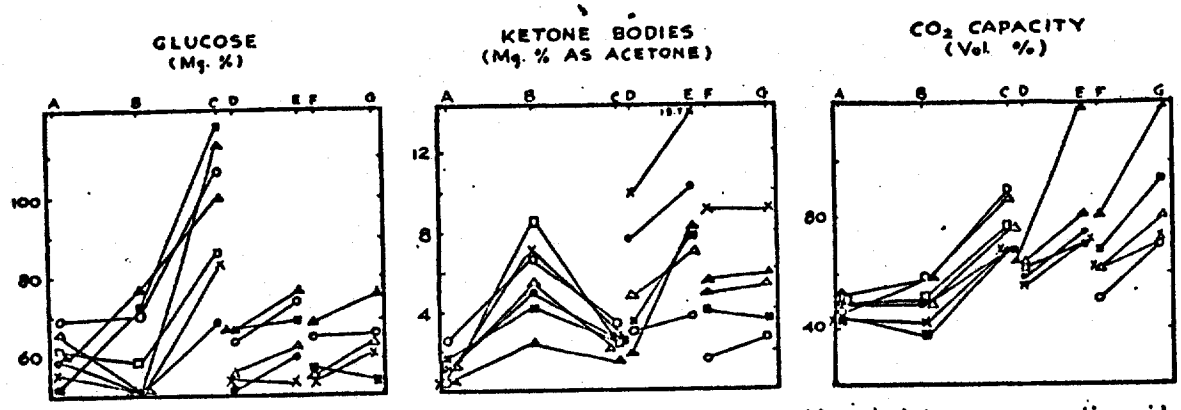

Fig. 7. Continuous oral *n*-butyric acid— + intravenous propionic acid— + intravenous acetic acid— + $NaHCO_3$

Hours of administration

48 | 22 | 6 | 18 | 6 | 18 | 6

A B C D E F G

<—Starved—><———— 0.5 mole *n*-butyric acid, 3.5 g KOH, 0.5 g NaOH/L (oral) ————>

<—0.375 mole—> propionic acid/L adjusted to pH 6.0 with NaOH (IV)

<—0.375 mole—> acetic acid/L adjusted to pH 6.0 with NaOH (IV)

<—0.375 mole—> $NaHCO_3$/L (IV)

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B Be	B-C Be	B-C Pv	C-D Be	D-E Be	D-E Av	E-F Be	F-G Be	F-G Nav
○ 1	220	40	(17)	1.39	1.25	1.87	1.23	1.23	1.87	1.23	1.23	1.87
● 2	142	63	D	1.15	1.21	1.19	1.27	1.15	1.19			
□ 3	363	41	D	1.63	1.65	1.83						
■ 4	316	37	(13)	1.47	1.46	2.03	1.15	1.32	2.03	1.46	1.55	2.03
△ 5	347	31	D	1.78	1.82	2.42	1.23	1.29	2.42	1.24	1.29	2.42
▲ 6	377	57	D	1.17	1.17	1.33	1.09	1.09	1.33	1.12	1.12	1.33
X 7	378	59	D	1.24	1.25	1.01	1.13	1.13	1.01	1.33	1.34	1.01

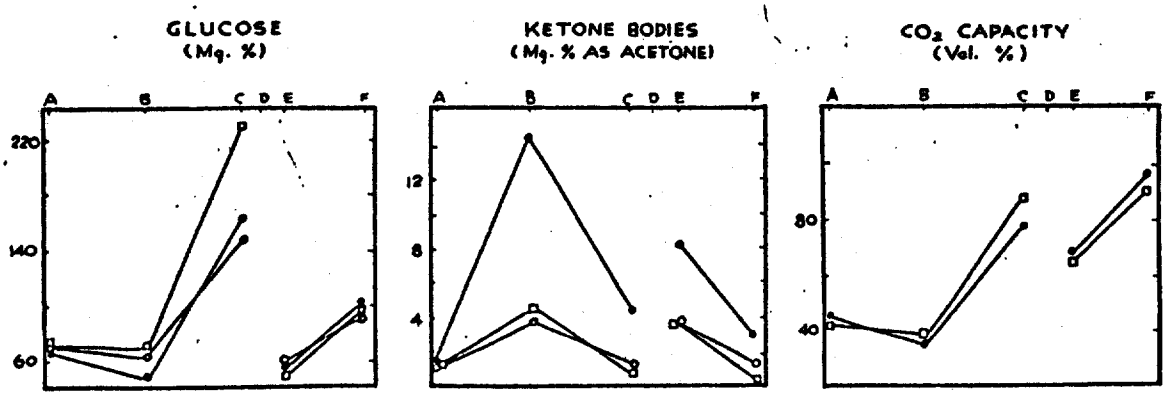

Fig. 8. Continuous oral *n*-butyric acid — + intravenous glucose — + intravenous propionic acid

Hours of administration

29 | 17 | 6 | 25 | 17 | 6

A B C D E F

<—Starved—><———— 0.5 mole *n*-butyric acid/L (oral) ————><—Starved—><———— 0.5 mole *n*-butyric acid/L (oral) ————>

<—0.1875 mole glucose, 0.375 mole $NaHCO_3$/L (IV)—>

<—0.375 mole propionic acid/L adjusted to pH 6.0 with NaOH (IV)—>

Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B Be	B-C Be	B-C Gv	D-E Be	E-F Be	E-F Pv
○ 1*	142	63	D	1.16	1.16	0.60	1.13	1.19	1.20
● 2	378	59	(1)	1.25	1.25	0.64	1.22	1.27	1.28
□ 3	316	37	[5]	1.29	1.24	0.96	1.07	1.33	1.92

* No bicarbonate added to glucose.

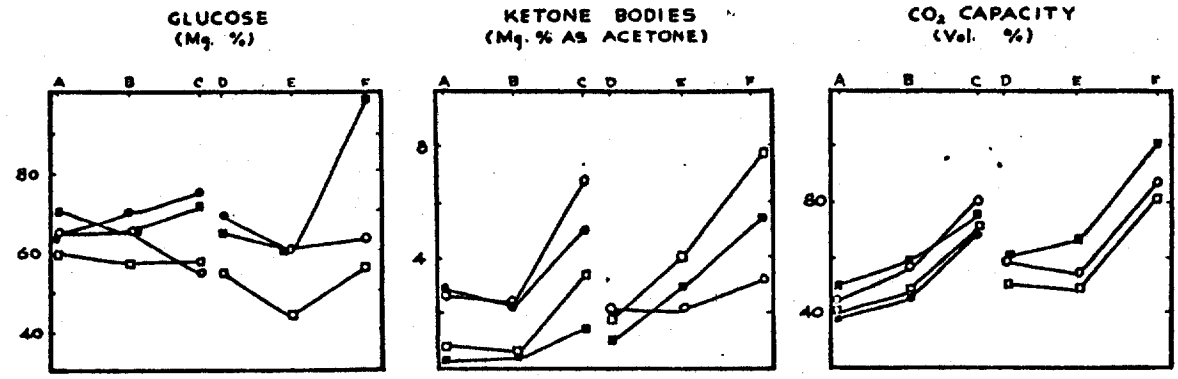

Fig. 9. Continuous oral acetic acid — + intravenous *n*-butyric acid — + intravenous acetic acid

Hours of administration

52 | 20 | 6 | 22 | 20 | 6

A B C D E F

<—Starved—><————1 mole acetic acid, 8 g KOH, 1 g NaOH/L (oral)><—Starved—><————1 mole acetic acid, 8 g KOH, 1 g NaOH/L (oral)>

<—0.4 mole *n*-butyric acid/L adjusted to pH 6.0 with NaOH (IV)—>

<—0.8 mole acetic acid/L adjusted to pH 6.0 with NaOH (IV)—>

	Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B A_e	B-C A_e	B-C B_v	D-E A_e	E-F A_e	E-F A_v
○	1	441	48	D	2.74	2.32	1.66	2.60	2.50	3.22
●	2	440	42	D	2.40	2.26	1.90			
□	3	377	57	D	2.56	2.10	1.41	2.27	2:20	2.82
■	4	438	59	D	1.88	1.35	1.35	2.31	2.22	2.70

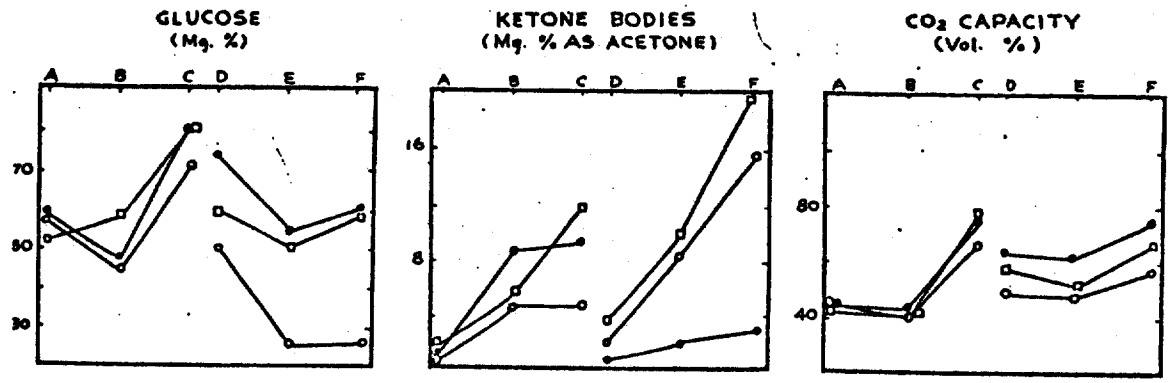

Fig. 10. Continuous oral acetic acid — + intravenous acetic acid — + intravenous *n*-butyric acid

Hours of administration

52 | 17 | 6 | 25 | 17 | 6

A B C D E F

<—Starved—><————0.75 mole acetic acid/L (oral)><—Starved—><————0.75 mole acetic acid/L (oral)>

<—0.4 mole acetic acid/L adjusted to pH 6.0 with NaOH (IV)—>

<—0.2 mole *n*-butyric acid/L adjusted to pH 6.0 with NaOH (IV)—>

	Run	Goat no.	Weight, kg	Stage of lactation or pregnancy	Rate of administration A-B A_e	B-C A_e	B-C A_v	D-E A_e	E-F A_e	E-F B_v
○	1	377	57	[6]	1.54	1.54	1.41	1.62	1.46	0.70
●	2	385	24	[8]	2.51	2.51	1.64	2.02	2.51	0.82
□	3*	363	41	D	2.60	2.60	3.91	2.59	2.59	1.95

* Intravenous solutions were 0.8 molar and 0.4 molar.

As both acetic and *n*-butyric acids may form acetyl CoA fragments for oxidation via the citric acid cycle and as they may both form ketone bodies if this cycle is blocked or overloaded, the ketogenic effects of the individual acids should reinforce one another. Figures 4 ($P<0.05$,* 6 ($P<0.002$,† 7 ($P<0.002$,‡ 9, and 10 clearly demonstrate this reinforcement. The experiments summarized in figures 9 and 10 suggest that when the citric acid cycle is overloaded, two molecules of acetate may not be significantly inferior to one molecule of *n*-butyrate in ability to produce ketone bodies.

C. Effect of propionic acid. In other animals propionic acid does not form ketone bodies, since it may be converted to glucose or glycogen or it may be catabolized via the citric acid cycle, for which it can supply the necessary oxaloacetic acid as well as the acetyl CoA.[11] This is also true for ruminants.[9,17,18,25,26] Figures 2 and 5 summarize confirming experiments. Schultz[24] has shown sodium propionate to be useful in the treatment of clinical ketosis.

Because of its ability to supply the oxaloacetate necessary for the operation of the citric acid cycle, propionic acid should facilitate the complete oxidation of acetic and *n*-butyric acids. Jarrett *et al.*[9] demonstrated the ketolytic effect of propionic acid given concurrently with acetic acid but failed to observe a similar effect with *n*-butyric acid. That propionic acid inhibits the ketogenic action of *n*-butyric acid is demonstrated by figures 1, 5 ($P<0.06$,§ 7 ($P<0.001$),# and 8.

If *n*-butyric and propionic acids are given to the animal in sufficiently large quantities, there will be a rise in blood

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$^{*}\frac{(B\text{-}A) - (D\text{-}C)}{(B\text{-}A)}$, $t = 2.67$, $n\text{-}1 = 6$.

$^{\dagger}\frac{(D\text{-}C) - (B\text{-}A)}{(D\text{-}C)}$, $t = 12.8$, $n\text{-}1 = 3$.

$^{\ddagger}\frac{E\text{-}F}{E}$, $t = 7.18$, $n\text{-}1 = 4$.

$^{\S}\frac{(B\text{-}A) - (D\text{-}C)}{(B\text{-}A)}$, $t = 2.70$, $n\text{-}1 = 4$.

$^{\#}\frac{B\text{-}C}{B\text{-}A}$, $t = 11.2$, $n\text{-}1 = 6$.

ketone bodies despite the presence of the propionate, as the amount of *n*-butyrate exceeds that which can be oxidized via the citric acid cycle or stored as fat under even the best conditions. This is shown in figure 2.

CHANGES IN BLOOD GLUCOSE LEVEL

A. Effect of propionic acid. It is commonly recognized that propionic acid is an efficient source of glucose.[11] This is true in ruminants as well as other animals.[9,20,21,23,25] Figures 1, 2 and 5, summarizing experiments in which propionic acid was given alone, and figures 5, 7, and 8, summarizing experiments in which propionic acid was given in combination with other acids, likewise confirm this effect.

B. Effect of n-butyric acid. Although in nonruminants *n*-butyric acid is not considered to be glucogenic, there is a known mechanism (*n*-butyrate —>acetoacetate—>acetone —>pyruvate) by which glucose may be formed.[11] The work which has been done on ruminants is contradictory. Schultz and Smith[25] found that when *n-butyric* acid was injected into the rumen of goats there was a brief initial increase in blood glucose level followed by a depression to less than one-half that of the initial level. They also found that caproic, caprylic, and capric acids caused similar decreases in blood glucose, the degree of change being inversely related to the rise in ketone bodies. Acetone, sodium and ethyl acetoacetate, or beta-hydroxybutyric acid did not have a comparable effect.

On the other hand, Jarrett, Potter, and Filsell[9] found that when 5 millimoles of sodium *n*-butyrate were administered intravenously to sheep over a period of 5 to 10 minutes, a marked rise in blood glucose occurred. They noted, however, that the animals responded with marked symptoms of distress and suggested that the rise may be due to glycogenolysis brought about by the sympatho-adrenal system. In a later paper Potter[21] reported that intravenous sodium *n*-butyrate relieved insulin convulsions in lambs and caused a rise in blood glucose greater than that produced by propionate, while acetate neither gave relief from the convulsions nor caused a rise in blood glucose. Convulsions, occurring in two adult ewes whose splanchnic nerves had been severed, responded well to

n-butyrate and propionate but not to acetate. He concluded that *n*-butyric acid may be a precursor of glucose. In interpreting experiments in which the quantity of radioactive carbon was determined in the lactose, casein, and fat of the milk secreted by cows which had received labeled *n*-butyrate, Kleiber *et al.*[12] concluded that a net synthesis of lactose had occurred. This very interesting work does not, however, indicate the mechanism by which this could take place, and other interpretations of the results are possible.

In the experiments here reported, the slow, continuous administration avoided any apparent alarm reaction except when anoxia, as evidenced by an extremely high CO_2 capacity, appeared to be intervening. The experiments involving oral administration of *n*-butyric acid are summarized in figures 1, 4, 7, and 8. The ketone values obtained in these experiments are lower than those attained by Schultz and Smith. Although there are exceptions, the results tend to confirm the finding that the drop in glucose level varies inversely with the degree of ketonemia. The experiments involving intravenous sodium *n*-butyrate (figures 5 and 6) reveal no consistent changes in blood glucose level.

C. Effect of acetic acid. The consensus of opinion is that in ruminants,[8,9,21,25] as in other animals,[11] acetic acid is not a precursor of glucose. The detection by Kleiber *et al.*[14] of radioactive carbon in the lactose and casein of the milk of cows given labeled acetate is readily explained as resulting from participation in Krebs' citric acid cycle rather than as net synthesis.[5] The experiments involving the oral administration of acetic acid (figures 3, 4, 9, and 10) show no consistent influence upon blood glucose level. Those involving the intravenous injection of sodium acetate (figure 6) likewise show no meaningful changes. The experiments summarized in figures 9 and 10 indicate that the sodium ion necessarily introduced in intravenous experiments has a marked effect upon the blood glucose level.

As the blood glucose level is sensitive to many factors, these experiments are not sufficiently well designed to reveal whether *n*-butyric or acetic acid has a minor effect upon blood

glucose level, but they do indicate that these two acids are in no way comparable to propionic acid as precursors of glucose.

SUMMARY

The effects of the administration of acetic, propionic, and *n*-butyric acids upon the blood glucose and ketone body levels of goats are consistent with the generally accepted mechanisms of metabolism.

1. Acetic acid and *n*-butyric acid form ketone bodies.
2. Propionic acid counteracts the formation of ketone bodies.
3. Propionic acid causes a marked increase in blood glucose level.
4. The experiments were not suitable for the observatior of slight effects upon the blood glucose level, but they clearly demonstrate that acetic acid and *n*-butyric acid do not caus an elevation of the blood glucose level comparable to tha caused by propionic acid.

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Propionate Metabolism by Slices of Mammary Gland and Liver of Lactating Rat*

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Lipogenesis from propionate-1-C^{14} was first reported by Feller and Feist (2) in slices of liver and adipose tissue. Favarger and Gerlach (3, 4) and Masoro and Porter (5) recently studied the incorporation of propionate-1-C^{14} into fatty acids of rat and mouse adipose tissue *in vivo* and *in vitro*. The incorporation of propionate, compared with that of acetate, was quite low. Favarger and Gerlach (4), on degrading the fatty acids formed from propionate, found labeling to be mainly in pentadecanoic and heptadecanoic acids, with propionate providing the terminal methyl 3-carbon unit. James, Peeters, and Lauryssens (6) found, after perfusion of cow udder with propionate-1-C^{14}, a small amount of labeled odd- and even-numbered carbon fatty acids in butterfat. Synthesis of odd-numbered carbon acids from propionate by a cell-free fat-synthesizing enzyme preparation has been reported (7).

We have studied propionate metabolism by slices prepared from the mammary glands and livers of lactating and nonlactating rats. In this paper, studies on lipogenesis from, and the oxidation of, C^{14}-labeled propionate and succinate by preparations from lactating rats are described.

EXPERIMENTAL PROCEDURE

Incubation—Mammary glands and livers of lactating rats of the Addis-Slonaker strain were used. The rats were decapitated, their livers or inguinal-abdominal glands were removed, and slices approximately 0.5 mm thick were prepared with a McIlvain-Buddle chopper (8). The slices (200 to 500 mg) were rinsed three or four times and incubated in 5 to 10 times their volume of Krebs-Henseleit bicarbonate buffer in an atmosphere of 95% O_2-5% CO_2 for 2 to 3 hours (9). The incubation vessels were 50 ml-Erlenmeyer flasks provided with center wells for CO_2 collection and closed with rubber serum sleeve caps (9). At the end of the incubation period, alkali was injected into the center well, and enough acid into the medium to liberate the CO_2. The tissue was denatured by a short period of heating (9).

Fractionation of Products—The fractionation procedure was essentially as described by Katz and Chaikoff (9). The slices were separated from the medium by centrifugation, and the tissue was washed three to four times with a small volume of water. The medium and combined washings (10 to 12 ml) constitute the aqueous extract. The slices were extracted exhaustively with alcohol-ether, and the extract was evaporated to dryness. The lipid residue was taken up in 10 ml of hexane and 5 ml of water, and the water phase was rejected (9). The tissue residue remaining after extraction of lipids was dissolved in 2 ml of 90% formic acid and 0.2 to 0.4 ml of 30% hydrogen peroxide. (This mixture dissolves most dry tissues in the cold.) Thus four major fractions were obtained: Fraction A, CO_2; Fraction B, water-soluble compounds; Fraction C, total lipid; and Fraction D, residue (mainly protein).

An aliquot of the aqueous extract (Fraction B above) was electrolytically desalted and chromatographed as described by Katz and Chaikoff (9). Water-saturated phenol and butanol-glacial acetic acid-water (100:22:50) were used as solvents. Radioautographs of these chromatograms were prepared on Kodak "no screen" x-ray film (9). Another aliquot of the aqueous extract was steam-distilled (9). The distillate contained the unused substrate, propionate or acetate, and the nonvolatile fraction, the water-soluble products.

An aliquot of the washed hexane solution containing the lipids was taken to dryness, and the fat was saponified with alcoholic KOH. After evaporation of the alcohol, the soap solution was acidified and extracted with ether. The fatty acids were re-extracted from the ether into dilute alkali, and the solution was made up to volume. Since there was no activity in either glycerol or unsaponifable lipids, these fractions were not studied.

Chromatography of Fatty Acids—Reversed phase column chromatography according to the method of Howard and Martin (10) on silicone-coated Celite was used to resolve the fatty acid mixtures. Paraffin oil was used as the stationary phase, and aqueous acetone, 35 to 75%, was used as the eluent.[1]

For application on the column, the fatty acids were re-extracted into ether; the ether was dried thoroughly with sodium sulfate and evaporated to a few milliliters. Approximately 200 mg of silica, 0.1 ml of paraffin oil, and a mixture of carrier acids (approximately 10 μmoles each of either the odd- or even-carbon series) were added, and the ether was evaporated.[2] The mix-

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[1] In our hands, procedures for column preparation as described in the original paper (10) gave tightly packed, impermeable columns. To prepare rapidly flowing columns, the slurry of siliconized silica and paraffin oil in 75% acetone was poured into a tall cylinder and freed from rapidly settling particles by decantation. The slurry was added to the column under the surface of the liquid 5 to 10 ml at a time and permitted to settle before the next addition was made. Ten-gram columns 1 cm in diameter retained a flow rate of approximately 100 ml per hour under 3 to 5 pounds of pressure. Such columns are usable for more than 35 runs.

[2] Anhydrous conditions during the evaporation of the ether are essential to avoid losses of C_7 to C_{10} acids. These acids are volatile with water vapor and will be lost if the mixture is not kept dry.

ire was slurried with approximately 5 ml of 35% acetone and applied to the column. The acids were eluted with increasing concentrations of aqueous acetone as indicated in Fig. 1. Fractions of 3 to 6 ml were collected and titrated with 0.02 N NaOH with a microburette.

Such a column resolves fatty acid mixtures of from 7 to 18 carbon atoms. Resolution of the odd-carbon and preceding even-carbon acid pair is not always complete, and with the shorter acids overlapping may occur. The resolution of adjacent odd- and even-carbon acids increases with chain length. C_8 and C_9[3] are difficult to resolve, but adequate separations of C_{10} and C_{11} were obtained (Fig. 1). In a mixture of C_{10} and C_{11} acids, the minor component can be estimated in the presence of a 10-fold excess of the adjacent acid.

Gas Chromatography—A Beckman GC-2 instrument with a thermal conductivity detector and a Bristol recorder of 10-mvolt sensitivity was used. The free (unesterified) acids were chromatographed according to the method of Metcalfe (11) on a column (0.5 × 90 cm) packed with 25% LAC 2 R-446 (diethylene glycol adipate polyester) and 2% phosphoric acid in Celite 545. The column temperature was initially 160° and was increased to 190° after C_8 emerged, and to 220° after C_9. To collect the emerging fatty acids, a Luerlock syringe adapter was welded to the gas exit line. The line was heated to 250°. The gas was passed out through 12-cm No. 18 hypodermic needles, which were dipped into tubes containing 5 ml of dilute alkali. Tubes and needles were changed manually, and samples were collected from the emergence of a peak to the onset of the next one.

The radioactive fatty acids up to 12 carbon atoms were separated from the large excess of higher fatty acids by column chromatography, and the acids were extracted into ether. Unlabeled carrier acids, 1 to 2 mg each from acetic to lauric acids, were added, and the dried ether solution was carefully concentrated to approximately 0.05 ml and injected into the column. When known mixtures of even-carbon C^{14} acids were analyzed, a few per cent of the radioactive acids appeared in the adjacent odd-carbon acid peaks, probably owing to the difficulty in the clean separation of fractions.

C^{14} Assay—The radioactivity was assayed with a Nuclear-Chicago gas flow counter with a Mylar window. CO_2 was precipitated as barium carbonate on glass filter paper. Carrier carbonate to yield 10 to 20 mg of precipitate was added. Suitable self-absorption corrections, to correct to zero mass, were applied. The other fractions were assayed by evaporating small aliquots (0.05 to 0.2 ml) of the solutions on aluminum disks. Carrier propionate was added as required.[4] The radioactivity in the compounds on the chromatograms was assayed directly on the paper (9) with a 6-cm gas flow Geiger tube with a window of thin aluminized Mylar. The C^{14} in the fractions was expressed either as per cent of the initial substrate C^{14} or as per cent of utilized C^{14} (utilized C^{14} = initial substrate C^{14} − C^{14} in volatile fraction).

[3] Fatty acids will henceforth be designated as C_8, C_7, etc., the subscript referring to the number of carbon atoms in the acid chain.

[4] When aliquots containing propionate at very low concentrations evaporate, erratic and large activity losses occur even with alkaline solutions. For radioassay, solutions were adjusted to be 0.02 to 0.05 M in sodium propionate and to approximately 0.005 M in alkali. For counting, aliquots of up to 0.5 ml evaporated with proper technique on 1.5-inch disks do not require self-absorption corrections; such platings are stable overnight.

RESULTS

Lipogenesis from Propionate—The distribution of the labeled products from carboxyl-labeled propionate and acetate in mammary gland and liver slices is shown in Table I. In the absence of glucose, the major radioactive product from pro-

TABLE I

Metabolism of carboxyl-labeled propionate and acetate by slices of mammary gland and liver of lactating rats

Slices were incubated for 3 hours in 4 ml of Krebs-Henseleit bicarbonate buffer in an atmosphere of 95% O_2-5% CO_2. Either 400 or 500 mg of slices were used in the different experiments. Glucose concentration, when used was 2 mg per ml, and that of insulin, 0.1 unit per ml. The specific activity of the labeled substrates was approximately 0.5 μc per μmole.

Rat No.	Substrate: Label	Substrate: Concentration*	Addition: Glucose	Addition: Insulin	Percentage of C^{14} in: CO_2	Lipid	Residue	Water-soluble: Non-volatile	Water-soluble: Volatile	Recovery
										%
Mammary gland										
8	Acetate-1-C^{14}	10	+	−	13	68		20		101
	Propionate-1-C^{14}	10	+	−	30	33		41		104
10	Acetate-1-C^{14}	10	−	−	12	3	2	83		100
		10	+	−	10	73	3	12		98
		10	+	+	7	77	2	6		92
	Propionate-1-C^{14}	10	−	−	19	0.1	0.9	77		97
		10	+	−	32	34	2	28		96
		10	+	+	25	44	2	25		96
9	Propionate-1-C^{14}	10	−	−	26	0.1	2	6	56	90
		10	+	−	37	10	3	7	32	89
		10	+	+	31	10	2	7	35	85
		3.3	+	+	55	16	3	8	12	94
9B	Propionate-1-C^{14}	3.3	−	−	29	0.2	2	4	63	98
		3.3	+	−	37	34	2	6	16	95
		3.3	+	+	34	32	2	4	22	94
11	Propionate-1-C^{14}	10	+	+	16	17	2	2	55	92
		50	+	−	5.5	2.1	0.5	0.6	82	94
		50	+	+	5.6	3.3	0.5	0.6	86	92
Liver										
9	Propionate-1-C^{14}	10	−	−	67	0.4	0.7	20	0.2	89
		10	+	+	68	0.3	0.6	19	0.3	89
9B	Propionate-1-C^{14}	3.3	+	+	74	0.3	0	12	2	88
11	Propionate-1-C^{14}	10	+	−	61	3.4	3	31		98

* Expressed as micromoles per incubation vessel.

TABLE II

Metabolism of propionate-1-, 2-, and 3-C^{14} by slices of mammary gland

In Experiment 5, each vessel contained 200 mg of slices in 2 ml of buffer; all others, 400 mg in 4 ml. Other conditions were as in Table I.

Rat No.	Propionate		Glucose	Percentage of C^{14} in					Recovery
	Concentration*	Label		CO_2	Lipid	Residue	Water-soluble		
							Non-volatile	Volatile	
									%
5	6	1-C^{14}	+	32	37	1.9	1.8	19	92
		2-C^{14}	+	11	53	4.1	6.1	16	90
		3-C^{14}	+	10	46	4.6	6.2	34	101
11A	10	1-C^{14}	+	7.5	5.9	0.6	1.0	77	92
		2-C^{14}	+	1.0	4.8	0.9	1.8	86	92
		3-C^{14}	+	1.3	6.0	1.0	2.1	87	97
11B	10	1-C^{14}	+	16	17	1.9	1.8	55	92
		2-C^{14}	+	3.8	15	2.9	3.8	68	90
		3-C^{14}	+	5.2	16	4.6	3.7	67	97
13	2	1-C^{14}	−	26	0	1.2	3.1	69	99
		2-C^{14}	−	6.1	0	0.8	6.9	82	96
		3-C^{14}	−	9.6	0	5.6	7.8	68	92
		1-C^{14}	+	27	34	2.2	3.0	25	91
		2-C^{14}	+	9.1	43	6.7	5.7	28	93
		3-C^{14}	+	9.9	45	8.3	4.8	22	90
14†	2	1-C^{14}	+	29	64	2.9	4.1		
		2-C^{14}	+	7.0	74	7.2	11		
		3-C^{14}	+	13	70	6.9	9.9		

* Expressed as micromoles per incubation vessel.

† Added C^{14} was not determined. Results are expressed as percentage of recovered C^{14}.

pionate-1-C^{14} was $C^{14}O_2$, and negligible incorporation into lipid was observed. Smaller amounts of C^{14} were incorporated into the nonvolatile water-soluble fraction and into the residue, mainly in glutamic acid (see below). In the presence of glucose, propionate was readily incorporated into lipids. Virtually all of the activity was recovered in fatty acids, and only trace amounts were found in the unsaponifiable fraction and glycerol. The necessity of glucose for lipogenesis from propionate parallels a similar requirement well known for lipogenesis from acetate. The addition of insulin did not affect lipogenesis at a low propionate level, but there was possibly a small stimulation at a higher propionate concentration. Under such conditions, glucose oxidation in the absence of insulin may become limiting. The incorporation of propionate into fatty acids was quite variable from animal to animal. The maximal incorporation of propionate into fatty acids was approximately 10 μmoles of propionate per g of mammary gland slices.

In mammary gland, propionate-1-C^{14} was oxidized to a larger extent than acetate-1-C^{14}, but incorporation into fatty acids was approximately half that of acetate. In livers of the lactating rat (Experiments 9 and 10), lipogenesis from propionate-1-C^{14} was only a fraction of 1%. Similar results with liver slices were reported by Landau *et al.* (12).

Results with propionate-1-C^{14} suggested that propionate was incorporated into fatty acids as a 3-carbon unit, yielding fatty acids containing an odd number of carbons. It appeared likely that propionate carbon could in addition be incorporated into fatty acids via a 2-carbon acetyl moiety. If this mechanism were to occur, a higher incorporation into fatty acids from propionate-2- and 3-C^{14} than from propionate-1-C^{14} would be expected. With propionate levels of 10 μmoles per vessel, no significant difference could be detected, but when propionate concentration was lower (2 μmoles per vessel) incorporation of propionate-2- and 3-C^{14} was approximately 15 to 20% higher than that of propionate-1-C^{14} (Table II).

Identification of Fatty Acids—The isotopic composition of these acids is presented in Fig. 1 and Table III. To identify the acids,

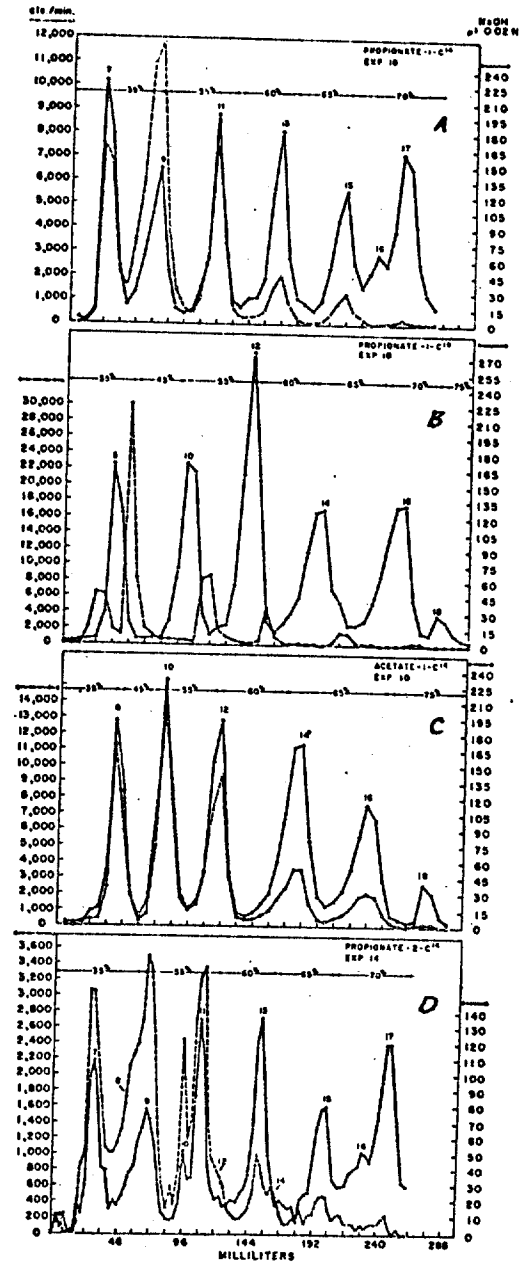

FIG. 1. Composition of the fatty acid fraction formed by rat mammary gland from propionate-1- and 2-C^{14} and acetate-1-C^{14}. In *A* and *D*, odd-carbon fatty acids from C_7 to C_{17} were added as carriers; in *B* and *C*, even-numbered carbon fatty acids from C_8 to C_{16}. The C_{16} peak in *A* and *D* is due to endogenous fatty acid. *Solid line*, titrable acids; *broken line*, radioactivity per sample. Samples of 6 ml were collected in *A* to *C*, and 3-ml samples were collected in *D*. Eluted with aqueous acetone solutions of increasing acetone content as indicated in the top line.

TABLE III

Activity distribution in fatty acids formed by rat mammary gland slices

Experiment No.	Substrate	Label	Carbons in fatty acid (activity in nonanoic or decanoic acids = 100)												Percentage of C^{14} in acids	
			7	8	9	10	11	12	13	14	15	16	17	18	Odd	Even
8	Propionate	1-C^{14}	94		100		9								100	
9	Propionate	1-C^{14}	81		100		36								100	
10	Propionate	1-C^{14}	52		100		47		13		10		1.5		100	
	Acetate	1-C^{14}		75		100		67		34		22		1.0		100
3	Glucose*	C^{14}		70		100		60		25						100
13	Propionate	2-C^{14}	44	25†	100	16	50	3	12	1	5				83	17
14	Propionate	1-C^{14}	31		100		45		19		10				100	
		2-C^{14}	53	25†	100	13	56	5	15	2	9				85	15
		3-C^{14}	50	25†	100	21	62	4	14	2	9				84	16
9–14‡	Propionate	1-C^{14}	39	2	100	2	18	0							97	3
		2-C^{14}	43	13	100	21	39	10							80	20

* Glucose was uniformly labeled. Approximately 4% of the fraction was in hexanoic and shorter chain acids.

† Separation from C_9 was not complete. The value represents an approximation.

‡ Pooled samples were analyzed by gas chromatography. Preliminary separation in column from higher fatty acids was followed by gas chromatography.

they were cochromatographed with carrier odd- and even-carbon fatty acids. With propionate-1-C^{14}, the activity peaks always coincided with the odd-carbon acid peaks, and no significant C^{14} could be detected in the even-carbon acids. As shown in Table III, C_7, C_9, and C_{11} accounted for 80 to 90% of the counts in this fraction. The rest was in C_{13}, C_{15}, and sometimes in C_{17}, decreasing in activity with chain length. The identity of the acids was confirmed by gas chromatography. On the Celite columns, acids shorter than C_7 emerge together with C_7. By gas chromatography, however, a very small amount of C^{14} was detected in valeric acid, but none in shorter acids. It appears that insulin had no effect on the fatty acid pattern.

When fatty acids from acetate-C^{14} were analyzed, it was found that C_8 and C_{10} contained most of the activity. The C^{14} distribution was quite similar to that found by Abraham, Matthes, and Chaikoff (13) in their mammary gland preparations. The pattern obtained with uniformly labeled glucose-C^{14} resembled that from acetate-C^{14}, except that a few per cent of labeled caprylic and possibly shorter acids were detected (Table III).

When fatty acids from propionate-2- and 3-C^{14} were chromatographed, the distribution of C^{14} resembled largely that with propionate-1-C^{14}. When fractional incorporation of propionate into lipids was low (at high substrate concentrations, Experiment 11, Table II), less than 10% appeared to be present in even-carbon acids, and the extent of the incorporation could not be estimated reliably. However, with high fractional incorporation at low substrate concentrations (Experiments 13 and 14), capric and caproic acid each were found to contain 5 to 10% of the total activity, and smaller amounts were detected in lauric and myristic acids. Approximately 15 to 20% of the total activity in the fraction was in even-carbon fatty acids. Also, as detected by gas chromatography, approximately 20% of the total radioactivity was in the even-carbon fatty acids. The small amount of even-carbon acids detected by gas chromatography in the pooled fatty acids from propionate-1-C^{14} is of doubtful significance, and it appears that very little, if any, such fatty acids were formed from propionate-1-C^{14}.

CO_2 Production—Oxidation of the carboxyl carbon of propionate is more extensive than that of acetate (Table II). The $C^{14}O_2$ yield from propionate-1-C^{14} was 3 to 5 times higher than from propionate-2- and 3-C^{14}. Similar findings have been reported by Landau *et al.* (12). Table II shows that the incorporation of propionate-2- and 3-C^{14} into the nonvolatile fraction and residue is 2 to 3 times higher than that of propionate-1-C^{14}. In some experiments (Table II), it appeared that the $C^{14}O_2$ formation from carbon 3 of propionate was more extensive than from carbon 2, but the data were too limited for a definite conclusion. To obtain more reliable information on this point, the $C^{14}O_2$ yields from propionate-2 and 3-C^{14} in the absence of glucose were compared in a series of experiments with mammary glands of six rats. Each determination was performed in triplicate. Although the $C^{14}O_2$ yield from carbon 3 was on the average somewhat higher than the yield from carbon 2, the difference did not exceed 10% and was statistically not significant.

Water-soluble Products and Residue—The incorporation of labeled propionate into water-soluble products by liver slices was several times greater than that by mammary gland slices. The C^{14} yield from propionate-2- and 3-C^{14} in water-soluble products and the residue appeared to be approximately equal and 3 to 5 times higher than that from propionate-1-C^{14}. The incorporation into the residue in the mammary gland in many cases exceeded that in the soluble nonvolatile fraction. The nature of the C^{14} in the residue of mammary gland was not investigated. In liver residue, it was shown that the C^{14} was present predominantly in glutamate and aspartate of the protein. It is probable that the C^{14} in the mammary gland residue also reflects protein incorporation of the dicarboxylic amino acids.

The composition of the water-soluble fraction as brought out by paper chromatography is reported in Table IV and Fig. 2. In mammary gland slices incubated with propionate-C^{14}, the major labeled compounds were glutamine and glutamic acid, which accounted for two-thirds to over three-quarters of the activity in the fraction. Aspartic acid contained approximately 5 to 10% of the activity, and smaller amounts were found in alanine, lactate, malate, succinate, and fumarate.[5] The patterns

[5] The total incorporation into the water-soluble fraction was low, and the fraction of the substrate appearing in any of the compounds, except glutamic acid and glutamine, ranged from 0.1 to

TABLE IV

Metabolism of propionate-C^{14} and succinate-C^{14} by slices of mammary gland and liver of lactating rats

The slices (400 mg) were incubated for 3 hours with 4 ml of buffer containing 2 mg of glucose per ml. Each flask contained 5 μmoles of propionate and 1 μmole of succinate; one substrate was labeled; one, unlabeled. The results are expressed as per cent of the utilized C^{14} (utilized C^{14} = recovered C^{14} − C^{14} remaining in substrate). The specific activity of propionate-1-C^{14} was approximately 1 μc per μmole; that of other substrates, approximately 0.5 μc per μmole.

	Rat 1				Rat 2			
	Mammary gland		Liver		Mammary gland		Liver	
	Propionate-1-C^{14}	Succinate-1,4-C^{14}	Propionate-1-C^{14}	Succinate-1,4-C^{14}	Propionate-2-C^{14}	Succinate-2,3-C^{14}	Propionate-2-C^{14}	Succinate-2,3-C^{14}
Per cent of added C^{14} utilized	82	94	93	95	17	89	61	89
Substrate utilized (μmoles)	4.1	0.94	4.6	0.95	0.85	0.89	3.5	0.89
Per cent of utilized C^{14} in:								
CO_2	38	15	71	15	10	0.5	25	5.6
Lipid	49	0.9	1.1	0.6	65	2.0	3.9	1.4
Residue	2.9	0.6	5.9	2.9	5.0	3.1	2.0	4.0
Water-soluble products	5.9	83	21	82	20	94	69	90
Glutamate*	4	0.5	3	0.4	19		5	2
Aspartate	0.4	4	0.9	1	0.4	4	3	3
Glucose			9	0.8			28	1
Lactate + alanine	0.6	0.6	5	2	0.8	4	22	10
Malate	0.1	68	1	65		83	8	69
Succinate†	0.3		1				3	
Fumarate	Trace	9.1	0.2	9		2		5
Others‡	0.1	0.7	1	4		1		

* Contains glutamic acid, glutamine, and pyrrolidonecarboxylic acid.

† Methylmalonic acid is not resolved from succinic acid in this solvent mixture.

‡ Contains citric acid and unidentified compounds.

obtained with propionate-2- and 3-C^{14} were similar to those from propionate-1-C^{14}. No incorporation of propionate into glucose or lactose, which was synthesized extensively under our conditions, could be detected.

In liver slices of lactating rats, from 40 to 70% of the C^{14} in the water-soluble fraction was present in glucose, and another 10 to 30% was in lactate and alanine. Thus, from 60 to 80% of the soluble products were accounted for by triosephosphate derivatives, 10 to 20% were incorporated into glutamic acid and glutamine, and the rest (10 to 15%), into aspartate and the dicarboxylic acids. The pattern obtained from the three labeled propionates was similar.[6]

Metabolism of Succinate—Results reported above indicated that in mammary gland as well as liver, propionate enters the Krebs cycle and may yield acetyl groups by decarboxylation of oxaloacetate and pyruvate, and glutamate via α-ketoglutarate. To ascertain the operation of such a pathway, slices were incubated with labeled succinate; the results are reported in Table V. It was found that C^{14} incorporation by mammary gland slices into CO_2 and lipids from succinate was very low, one-tenth or less than that from propionate at similar substrate concentrations. This was not due to lack of penetration of succinate into the cell. Chromatograms of the aqueous fraction, presented in Fig. 2, and data of Tables IV and V reveal extensive metabolism of succinate and its conversion into malate. In some experiments, only traces of added succinate remained. From 60 to 80% of the recovered C^{14} was in malate. Some activity was found in fumarate and aspartate, and a little in citrate, glutamate, and a few other compounds. A few experiments were performed with citrate-2,5-C^{14} (Table V). Citrate was much more inert than succinate, possibly owing to the lack of penetration. However, at low concentrations (0.002 M), the fraction converted to malate was appreciable.

The oxidation of succinate was somewhat higher in liver slices than in the mammary gland, but succinate metabolism in its main features was similar to that with mammary gland. Lipogenesis and oxidation were small fractions of that from propionate. The most striking difference between propionate and succinate metabolism in liver slices was the virtual absence of labeled glucose among the products from succinate. As in mammary gland, the major product from succinate was malate, which appeared to accumulate (Table IV).

DISCUSSION

Established pathways of propionate metabolism are summarized below. Only the over-all transformation of the carbon chain is considered.

1. Synthesis of odd-carbon numbered fatty acids:

$$\overset{*}{C}H_3—\overset{*}{C}H_2—\overset{*}{C}OOH + n\ CH_2—COOH \rightarrow$$

$$\overset{*}{C}H_3—(\overset{*}{C}H_2)_2—(CH_2—CH_2)_{n-1}—CH_2—COOH$$

This synthesis has been demonstrated in adipose tissue by Favarger and Gerlach (3) and is a major pathway in rat mammary gland slices in the presence of glucose.

2. Succinate path:

Propionyl-CoA → methylmalonyl-CoA →

succinyl-CoA → Krebs cycle

Conversion of propionate to methylmalonate and succinate has been established in animal tissue by Katz and Chaikoff (14) and Flavin and Ochoa (15).

1%. At such levels, the results reported in Table IV are only semiquantitative, since the variability in C^{14} assay between duplicate chromatograms increased from ±10% to ±50% at very low C^{14} levels.

[6] The general pattern in liver slices from lactating rats was similar to that of nonlactating animals; however, liver slices from normal nonlactating rats incorporated much more propionate into succinate and less into glucose. The pattern of propionate metabolism in liver slices of lactating rats is very similar to that of diabetic rats or those deprived of food. Under all of these conditions, incorporation into glucose was high, and into succinate, relatively low. A study of propionate metabolism in normal and diabetic rats will be reported elsewhere.

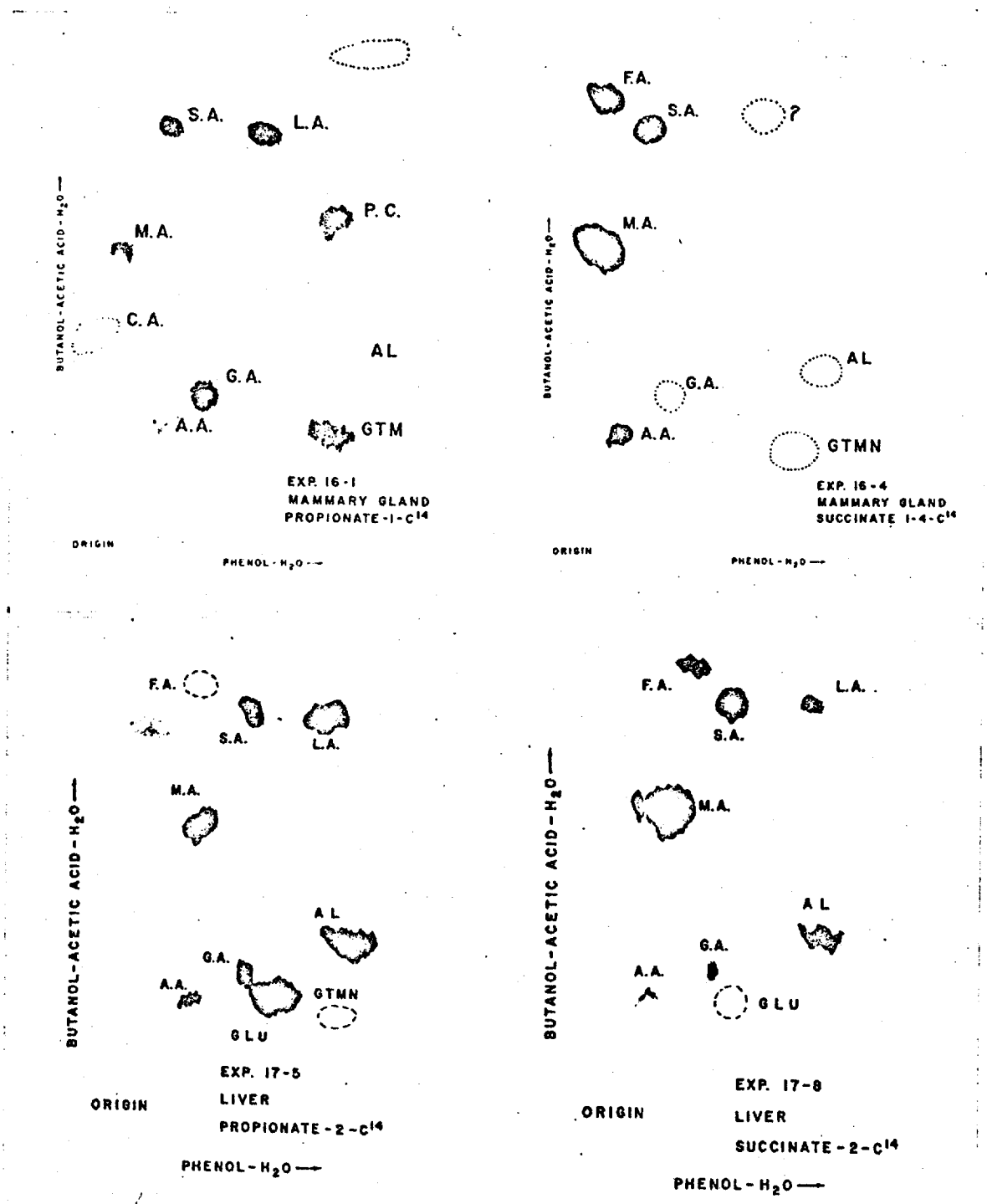

FIG. 2. Radioautographs of chromatograms of desalted extracts of mammary gland and liver slices incubated with C^{14}-propionate and succinate. *A.A.*, aspartic acid; *G.A.*, glutamic acid; *GTMN*, glutamine; *AL*, alanine; *GLU*, glucose; *P.C.*, pyrrolidonecarboxylic acid (probably artifact from glutamine); *M.A.*, malic acid; *S.A.*, succinic acid; *L.A.*, lactic acid; *F.A.*, fumaric acid; *C.A.*, citric acid.

3. Malonic semialdehyde path:

$$H_3C{-}CH_2{-}\overset{*}{C}OOH \rightarrow$$

$$OHC{-}CH_2{-}\overset{*}{C}OOH \begin{cases} \nearrow COOH{-}CH_3 + \overset{*}{C}O_2 \\ \searrow NH_2CH_2{-}CH_2{-}\overset{*}{C}OOH \end{cases}$$

The formation of malonic semialdehyde from propionate has been established by Rendina and Coon (16). In animal tissue it was shown to be aminated to β-alanine, a pathway of limited extent. In plant tissue, Giovanelli and Stumpf (17) found malonic semialdehyde to be oxidized to acetyl-CoA and CO_2, carbon 3 of propionate becoming the carboxyl of acetate.

4. Malonic acid pathway:

TABLE V

Metabolism of succinate and citrate-C^{14} by slices of mammary gland and liver of lactating rats

There were 5 μmoles of acid per vessel in Experiments 3 to 12 and 2 μmoles in Experiment 15. Conditions were as in Table I.

Experiment No.	Tissue	Substrate	Glucose	Percentage of C^{14} recovered in		
				CO_2	Lipid	Malate
3	Mammary gland	Succinate-2,3-C^{14}	+	0.3	1.6	86
8	Mammary gland	Succinate-2,3-C^{14}	+	0.2	0.5	77
12	Mammary gland	Succinate-1,4-C^{14}	+	3.0	0.06	54
		Succinate-1,4-C^{14}	−	3.5	0.01	72
		Succinate-2,3-C^{14}	+	0.3	0.8	49
		Succinate-2,3-C^{14}	−	0.8	0.01	76
		Citrate-2,5-C^{14}	+	1.2	0.01	15
		Citrate-2,5-C^{14}	−	0.3		6
15	Mammary gland	Succinate-1,4-C^{14}	+	3.0	0.2	80
		Succinate-1,4-C^{14}	−	3.8	0.02	67
		Succinate-2,3-C^{14}	+	0.5	1.8	53
		Succinate-2,3-C^{14}	−	0.6	0.1	70
		Citrate-2,5-C^{14}	+	1.9	0.1	20
		Citrate-2,5-C^{14}	−	2.4	0.1	29
	Liver	Succinate-1,4-C^{14}	+	12	0.1	65
		Succinate-2,3-C^{14}	+	3.8	0.6	67
		Citrate-2,5-C^{14}	+	13	0.1	15

$$CH_3—CH_2—\overset{*}{C}OCoA \rightarrow$$

$$COOH—CH_2—\overset{*}{C}OCoA \rightarrow CO_2 + CH_3\overset{*}{C}OCoA$$

The formation of malonyl-CoA from propionic acid was established by Vagelos and Earl (18) in *Clostridium kluyveri*. The malonic acid would yield an acetyl unit from carbon atoms 1 and 2. This pathway could explain synthesis of even-carbon fatty acids from propionate-1-C^{14}.

5. Conversion of propionate to pyruvate: such a pathway has been described in propionobacteria by Swick and Wood (19). It has not been shown to occur in animal tissues.

Fatty Acid Synthesis—In most mammalian tissues, synthesis of odd-carbon numbered fatty acids appears to be rather limited, and rat mammary gland seems to be an exception in this respect. Favarger and Gerlach (4) estimated that odd-carbon numbered fatty acids constitute from 0.25 to 0.5% of the carcass fat of mice. The incorporation of propionate into body fat *in vivo* and *in vitro* was reported (3–5) to be not greater than 5% that of acetate. In contrast, propionate incorporation into fatty acids by rat mammary gland slices is quite extensive and of the same order of magnitude as acetate.

The fatty acids synthesized from acetate or glucose contained mainly 8 to 12 carbons and presumably represent butterfat synthesis rather than tissue fat, which has a low proportion of acids of this chain length. Propionate incorporation proceeded predominantly into acids containing 1 carbon atom less, namely, from 7 to 11. Since the fatty acids were not degraded, the position of the label is not known unequivocally, but the degradation experiments of Favarger and Gerlach (4) and the results of Horning *et al.* (7) make it reasonable to assume that propionyl-CoA acts as acceptor for malonyl-CoA, and thus propionate carbon becomes the methyl end of the fatty acid.

James, Peeters, and Lauryssens (6) and Favarger and Gerlach (3–4) observed some formation of labeled even-carbon fatty acids from propionate-1-C^{14}. If such even-carbon acids were formed in our experiment, their fraction could not exceed 5%; this was the detection limit of our methods.

Oxidation of Propionate—The oxidative metabolic pathways of propionate in the mammary gland and liver have many common features. Our results show (*a*) that the oxidation of the carboxyl carbon exceeds greatly that of carbon atoms 2 and 3; (*b*) that a limited number of acetyl units was formed; and (*c*) that incorporation into soluble products and residue was several times higher from carbon atoms 2 and 3 than from carbon 1. Of special importance is the fact that there is little significant difference between the metabolic fates of carbon atoms 2 and 3. In liver slices of nonlactating rats, the metabolic fate of carbons 2 and 3 of propionate was also similar.[7]

These findings are consistent with the oxidation of propionate via succinate and the Krebs cycle. It appears that in liver slices of lactating rats, the major portion of the inflowing succinate was removed via oxaloacetate and pyruvate, yielding glucose, lactate, and alanine. In mammary gland, the major outflow was into glutamine and free and presumably protein-bound glutamic acid. Our results are not consistent with the major operation of other pathways, such as the malonic semialdehyde and the lactate pathway discussed above. With these pathways, the acetyl yield from propionate would be expected to be much larger, and the CO_2 yield from carbon atoms 2 and 3, markedly different. However, such pathways could occur to a limited extent.

Pathways of Propionate Utilization and Metabolism of Exogenous Succinate—Our results with succinate appear to be inconsistent with the conclusion that the oxidation of propionate, in both mammary gland and liver, proceeds via succinate. As brought out clearly in Tables IV and V, only small amounts of propionate were incorporated into malate, whereas exogenous succinate was predominantly oxidized to malate, which accumulated. Also, as shown in Table IV, the incorporation of propionate and succinate into dicarboxylic amino acids yielded a completely different pattern. In mammary gland, the glutamate to aspartate ratio was 10 and higher with propionate, but it was 0.1 to 0.5 with succinate. Also, with propionate, very little labeled fumarate was found, but with exogenous succinate, the incorporation into fumarate was appreciable, the activity in fumarate exceeding that in residual succinate.

In liver, the glucose yield from succinate was negligible, but this was a major product derived from propionate.

These findings may be interpreted to indicate that propionate oxidation does not proceed via succinate but rather by another pathway. However, our results, which indicate a similar fate of carbon atoms 2 and 3 of propionate, are hard to reconcile with pathways that exclude formation of symmetric dicarboxylic acids. We have been unable to adduce evidence for any alternate pathways in the mammary gland. Moreover, in liver, inhibition studies leave little doubt of the operation of the methylmalonate-succinate path.[7]

An alternate explanation for the observed inconsistency in the metabolism of propionate and succinate could be provided by a

[7] Unpublished observations.

hypothesis that the metabolic fates of endogenous and exogenous succinate are different. It may be postulated that exogenous succinate and its oxidation products, fumarate and malate, do not equilibrate with these acids when generated via the Krebs cycle, and that exogenous succinate is oxidized to malate by a system different from the Krebs cycle. It may be assumed that propionate and exogenous succinate are metabolized in different cells in the slice, each cell type permeable to only one substrate. For instance, propionate could be metabolized by undamaged tissue cells, which are impermeable to succinate. Damaged cells, however, such as may occur on the slice surface, may be permeable to succinate but unable to oxidize the acid beyond the malate state. Although this interpretation is more acceptable than the first, it should be stressed that no evidence to support such a hypothesis exists. At present, no definite conclusions are possible, and further study is required.

SUMMARY

1. In the presence of glucose, propionate-1-C^{14} was extensively incorporated into fatty acids by mammary gland slices of lactating rats.

2. Of the activity in this fraction, 80 to 90% was in heptanoic, nonanoic, and undecanoic acids; smaller amounts were in tridecanoic and pentadecanoic, and traces were in pentanoic and heptadecanoic acids.

3. The incorporation of propionate-2- and 3-C^{14} into fatty acids was somewhat larger than that of propionate-1-C^{14}. The composition of the fatty acids from all labels was similar, but those from the former also contained approximately 20% of even-carbon acids, mainly caproic and capric acids, and smaller amounts of even-carbon acids of longer chain length.

4. Propionate was extensively oxidized by mammary gland slices, the $C^{14}O_2$ yield from propionate-1-C^{14} being 3 to 4 times that of propionate-2,3-C^{14}. The $C^{14}O_2$ yield from propionate-2- and 3-C^{14} was approximately equal. The incorporation of the latter labels into proteins and water soluble products exceeded by several times that from propionate-1-C^{14}.

5. The water-soluble products formed from propionate-C^{14} were determined. In mammary gland, the major labeled compounds were glutamic acid and glutamine. There was little incorporation into dicarboxylic acids.

6. In liver slices, propionate was extensively metabolized. The major products were CO_2 and glucose. Smaller amounts were incorporated into lactate, alanine, and amino acids. The incorporation of propionate-1-C^{14} into fatty acids was quite low.

7. CO_2 formation and lipogenesis from succinate-C^{14} by mammary gland and liver slices was very low. The succinate was extensively oxidized to malate, which accumulated. The metabolism of citrate-C^{14} was sluggish, but the major product was malate. The incorporation of succinate into aspartate exceeded greatly that into glutamate. In liver slices, negligible amounts of glucose were formed from succinate.

8. The markedly different patterns of propionate and succinate metabolism appear to be inconsistent with the view that propionate is metabolized via methylmalonate, succinate, and the Krebs cycle. However, our results on propionate oxidation are consistent with propionate metabolism proceeding by the succinate pathway, and there is no evidence for an alternate pathway.

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PROPIONIC ACID

Propionic acid (propanoic acid (IUPAC)), CH_3CH_2COOH, is a water-white liquid with a sharp irritating odor resembling strong vinegar. It is freely soluble in water, alcohol, ether, chloroform, and other acids.

Propionic acid is found in dairy products such as milk, butter, and cheese, especially after fermentation or spoilage. It occurs in the vapors resulting from the carbonization of wood and coal, and at one time the distillates from charcoal production were the prime source of the acid. It also is found in the black liquor acids from sulfite pulp production.

Propionic acid finds use in the production of esters, salts, polymer products, and specialty chemicals.

Physical and Chemical Properties

Constants. The principal constants for propionic acid are shown in Table 1. Table 2 lists some binary azeotropes of propionic acid (1–5).

Table 1. Physical and Chemical Constants of Propionic Acid

melting point, °C	−22
boiling point, °C	141.1
vapor pressure, mm Hg, at 28.0°C	5
at 52.0°C	20
at 85.8°C	100
at 122.0°C	400
specific gravity, 20/4°C	0.992
20/20°C	0.9952
refractive index, n_D^{20}	1.3874
viscosity at 25°C, cP	1.035
surface tension in air at 20°C, dyn/cm	26.70
coefficient of cubical expansion at 0–133°C/°C	1.102×10^{-3}
critical temperature, °C	337.6
critical pressure, atm	53.0
specific heat at 20–137°C, cal/g	0.726
heat of fusion at melting point, cal/g	23.4
heat of vaporization at 139.3°C, cal/g	98.9
heat of combustion of liquid, cal/g	4931.5
flash point, Cleveland open cup, °F	150
weight at 20°C, lb/gal	8.28

Table 2. Properties of Binary Azeotropes of Propionic Acid at 760 mm Hg

Other component	Propionic acid, wt %	Bp, °C
water	4.5	99.2
pyridine	67.2	148.6
N,N-dimethylpropionamide	23.6	179.3
heptane	2	97.8
octane	24	120.9
nonane	54	134.3
decane	80.5	139.8

The system acetic acid–propionic acid also has been studied (6), as has that of acetic anhydride–propionic acid (7). In addition, propionic acid forms a ternary azeotrope with pyridine and undecane, boiling at 147.1°C, and containing 55.4% propionic acid and 26.4% pyridine (1).

Reactions. Propionic acid reactions are typical of those for a lower monocarboxylic acid such as acetic acid. Salts, amides, esters, the acid chloride, and the acid anhydride are formed in the usual way. Chlorination under ultraviolet radiation provides a mixture of chlorinated propionic acids (8). Refluxing the acid with aluminum alkyls, R_3Al, gives aluminum tripropionate, $Al(OOC_3H_5)_3$, as well as the partial intermediates $R_2Al(OOC_3H_5)$ and $RAl(OOC_3H_5)_2$ (9). Methyl propionate results from the reaction, at 270°C, with dimethyl ether, using activated carbon and phosphoric acid as catalysts (10). Shortwave ultraviolet irradiation causes the formation of dimers at the alpha methylene group (11). Gas-phase reaction with acetylene produces vinyl propionate (12,13) in a manner analogous to making vinyl acetate (see Vinyl compounds).

Manufacture

Formerly, the principal source of propionic acid was the liquid condensate secured from the manufacture of charcoal. This is a very minor source today. The oxo process (qv) is the major method of manufacture in the U.S. Ethylene and carbon monoxide are reacted under reductive conditions to produce propionaldehyde (qv), after which further oxidation converts the aldehyde to the acid (14–17). Large quantities of acid also are obtained from liquid-phase oxidation of propane (18), butane (19), mixed paraffins (20), and paraffin wax (21) (see Hydrocarbon oxidation). The oxo and hydrocarbon-oxidation routes are important both in the U.S. and in Europe. A further process is the direct oxidation of 1-propanol with nitric acid (22).

In the U.S. there are five commercial producers of propionic acid. In 1966, production was about 30 million lb, and this was about equally distributed between captive use and merchant sales. There were also several million pounds of imports. Producers and estimated capacities are listed in Table 3 (23), but oxo capacity is flexible and could be directed toward more propionaldehyde as required.

Table 3. Propionic Acid Producers, Capacities, and Processes

Producer	Capacity, million lb	Process
Celanese Chemical Company	10	hydrocarbon oxidation
Cliffs Dow Chemical Company	0.5	wood carbonization
Commercial Solvents Corporation	0.2	nitroparaffin by-product
Tennessee Eastman Company	10	oxo
Union Carbide Corporation	10	oxo

The price of propionic acid has declined through the last two decades as the newer processes, oxo and hydrocarbon-oxidation, have made more material available and new or expanded uses have developed. In 1955 propionic acid listed at 20.75¢/lb at the works; in 1967 the list price was 14.75¢/lb delivered. Expansions may cause a further lowering of costs and the savings can be passed on to the consumer. In addition, more acid is available from European sources and, with lower import duties, pressure on U.S. pricing may be greater than in the past.

Propionic acid is not classed as a flammable liquid by ICC regulations; however, it requires the normal handling precautions which would be accorded any organic compound of comparable flash point and vapor pressure. The acid usually is shipped in aluminum or stainless steel, or in steel drums with a polyethylene liner. Uncoated steel is not recommended.

Because of the widespread use of propionate salts in food products and animal feeds, the metabolism and toxicity of the acid and its salts have been studied intensively (24–26). The acid and its salts are considered to have low toxicity.

Analysis

The assay of a propionic acid sample may be determined by titration with standard base in the usual manner. The acid has been detected in weak solutions by paper chromatography (27), but usually it is measured by gas chromatography. Thus, it has been determined in mixed acids (28), in sewage (29), and in foods (30).

Uses

Propionic acid has relatively few commercial uses, and these require the propionate configuration to fit specific applications. In general, propionic acid outlets are limited by competition from acetic acid, which is available in vastly greater quantity and is cheaper. The acid has been used as a catalyst in the manufacture of polyester resins (31), and epoxy resins have been modified by esterification with the acid (32). The acid or its anhydride find a major use in the production of cellulose tripropionate and cellulose acetate propionate for plastics and fibers. Tung oil tripropionate is reported to impart antifungal properties to paint (33). An important outlet is the production of propionate salts by reaction with the corresponding carbonate or hydroxide (34). Both calcium and sodium propionates find extensive use as additives to inhibit mold and "rope" in baked goods; and as components of cattle and poultry feeds to prevent ketosis (35), as a fungistat (36), and to improve feed utilization (37,38). Ammonium propionate reportedly reduces viscosity and serves as a wetting agent in silver halide-gelatin emulsion systems in photography (39). Calcium propionate has been suggested as a scorch retarder for butyl rubber (40), and the zinc and cadmium salts serve as catalysts in making terephthalate polyesters (41). Vinyl propionate has received considerable study in the U.S. (42–44) and Europe (45–47) in copolymer systems for a variety of end uses.

A number of commercial weed-killing agents are derivatives of propionic acid, eg, 2-(2,4,5-trichlorophenoxy)ethyl 2,2-dichloropropionate, sodium 2,2-dichloropropionate, and 2-(2,4,5-trichlorophenoxy)propionic acid. (See Weed killers.) Various esters are useful in the flavor and perfume industries and in lacquers as special solvents. Methyl 2-cyclohexanyl propionate is used as a solvent in production of hydrogen peroxide from anthraquinhydrone (48); the benzyl ester is a perfuming agent for resinous products (49); *tert*-butyl propionate is claimed as an antidetonation improver in leaded gasoline (50); the vapors of the ethyl and butyl esters are antibacterial agents (51); and 2-chloroallyl propionate is an agent for washfast textile finishes (52). The octadecylamine salt is reported to be a molding assisting agent for silicate powder (53).

A probable use pattern of propionic acid, in 1967 in the U.S., is given in Table 4 (54).

OBSERVATIONS ON THE EFFECT OF CHEMICAL CONFIGURATION ON THE EDEMA-PRODUCING POTENCY OF ACIDS, ALDEHYDES, KETONES AND ALCOHOLS[1, 2]

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In an earlier publication (Finnegan *et al.*, 1947) we described a method for quantitatively evaluating the edema-producing potency on mucous membranes, of the irritants in tobacco smoke. Subsequently we have adapted this method to permit of its use in evaluating the edema-producing potencies of chemicals in water solution. The method and results obtained with four classes of chemical compounds are detailed below.

METHOD. Morphinized male albino rabbits of about 2 kgm. weight are used. One-half hour after morphinization (20 mgm. per kgm. body weight subcutaneously) the rabbit is tied in a supine position to an animal board. A pocket is made of the upper lid of the right eye and filled with the solution containing the irritant. The solution is held in place for 3 minutes, after which the lid is released and the rabbit returned to the upright position. One hour after application of the irritant the rabbit is sacrificed by cerebral concussion, the upper palpebral conjunctiva of each eye excised, weighed, dried in an oven to constant weight, and reweighed. From the wet and dry weights the ratio of moisture to dry weight for each membrane is calculated. The difference between the ratio for the right (exposed) eye tissue and that for the left (control) eye tissue represents the degree of edema production.

In the present study 5 rabbits were used at each molar concentration tested and 6 such groups in studying each irritant, except in a few instances where solubility was a limiting factor. With each irritant, the concentrations tested were so adjusted (solubility permitting) as to produce average moisture to dry weight gains (right eye ratio minus left eye ratio) spreading over a range up to about 5. Analysis of regression was then applied to the data to determine the equation for the best line to fit all points on the resulting curve. In this calculation the average moisture to dry weight gains were used as such, but the molar concentrations of the test material were converted to log units. From the resulting equation, we have for comparative purposes arbitrarily calculated the molar concentration that should produce a 2.5 unit increase in moisture to dry weight. The 2.5 point represents about a 50 per cent gain in moisture to dry weight as compared to the control tissue.

RESULTS AND DISCUSSION. *Acids.* The results obtained on the acids studied are summarized in table 1.

Among the monobasic organic acids, excepting formic, there is a tendency toward increasing edema-producing potency (EPP) with increasing molecular weight. Unsaturation increased EPP in the case of acrylic acid (compare with propionic) but not in the case of crotonic acid (compare with butyric).

The dibasic acid, succinic, has a lower EPP than the corresponding monobasic

[1] A preliminary report appeared in Fed. Proc., 8: 312, 1949.

[2] This investigation has been supported under a research grant from the American Tobacco Company.

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American Journal of Physiology. vol. 188(2) 1957

Metabolism of C^{13}-Carboxyl-Labeled Propionate by the Isolated, Perfused Dog Gastrocnemius[1]

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WITH THE TECHNICAL ASSISTANCE OF Joyce A. Swanson, Colleen Rehder and Ruth B. Deal

From the Department of Physiology, University of Minnesota Medical School, Minneapolis, Minnesota

ABSTRACT

LIFSON, NATHAN. (U. Minnesota Med. School, Minneapolis.) *Metabolism of C^{13}-carboxyl-labeled propionate by the isolated, perfused dog gastrocnemius.* Am. J. Physiol. 188(2): 227–232. 1957.—The metabolism of C^{13}-carboxyl-labeled propionate was studied in the isolated perfused dog gastrocnemius muscle, both resting and contracting. Under the conditions employed, 9–13% of the respiratory CO_2 was derived from the carboxyl carbon of propionate in the resting skeletal muscle preparation, and only 3–5% in the repetitively contracting gastrocnemius. Thus, during muscle activity, the carboxyl carbon of propionate contributed proportionally less to the metabolic mixture giving rise to CO_2 than during rest. Evidence was obtained for the conversion of carboxyl-labeled propionate to carboxyl-labeled lactate by isolated skeletal muscle, but not for its incorporation into muscle glycogen. When acetate and propionate are administered simultaneously to resting muscle, the rate of conversion of the carboxyl carbon of propionate to CO_2 is reduced to a much greater extent than that of the carboxyl carbon of acetate.

THIS PAPER reports a study of the conversion of C^{13}-carboxyl-labeled propionate to CO_2, lactate and glycogen by the isolated blood-perfused dog gastrocnemius muscle, either resting or contracting repetitively. Observations are also included on competition between propionate and acetate for conversion to CO_2 by resting muscle.

METHODS

Perfused Gastrocnemius. The experimental procedure was essentially as previously described (1). In brief, with the dog under sodium pentobarbital (Nembutal) anesthesia, the left gastrocnemius was isolated by dissection until its only connections with the body were via the continuations of the femoral artery and vein. The muscle was then transferred to a blood-oxygenator circuit for perfusion with heparinized blood taken from the same dog. The blood was aerated with CO_2-free oxygen for about 45 minutes before the muscle was inserted in the system and for about 30 more minutes with the muscle in place. The isotopic sodium propionate was then injected and the collection of respiratory CO_2 in sodium hydroxide absorbers was begun. The absorbers were changed at approximately hourly intervals. One modification of the previous apparatus consisted of use in all but a few of the experiments of a muscle chamber with a water jacket for thermostasis. In the experiments on resting muscles, 3 U of *d*-tubocurarine chloride were injected to prevent fasciculatory twitching of the muscle. In the experiments with contracting muscles, maximal stimuli were applied to the isolated sciatic nerve at a rate of 2/sec. beginning 10–25 minutes before administration of the propionate. In most of the experiments, 50,000 U of penicillin were injected to inhibit microbial growth. Control experiments on blood were performed by omitting the muscle from the perfusion system.

During the perfusion the muscle nearly always gained weight (1), and sometimes, possibly due to methemoglobin formation, the blood and muscle darkened in color. In a few instances, the darkening was very marked and the experiment was rejected without performing any analyses on the samples. No obvious relationship has been noted between the results to be reported and the degree of weight or color change.

Chemical Procedures. C^{13}-carboxyl-labeled propionic acid was synthesized by the Grignard method (2). Its partition coefficient agreed with that of reagent propionic acid. In the various batches of propionate used the C^{13} ranged from 1.25 to 3.25 atom % excess in the whole molecule. Initial propionate concentrations in the perfusion system were calculated on the assumption that blood plus muscle consisted of 75% water and that the administered compound distributed itself

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[1] This investigation was supported by grants from the Committee on Growth, National Research Council and from the National Institutes of Health (G3483).

uniformly in this water. Analytical procedures were as previously described (1).

RESULTS AND DISCUSSION

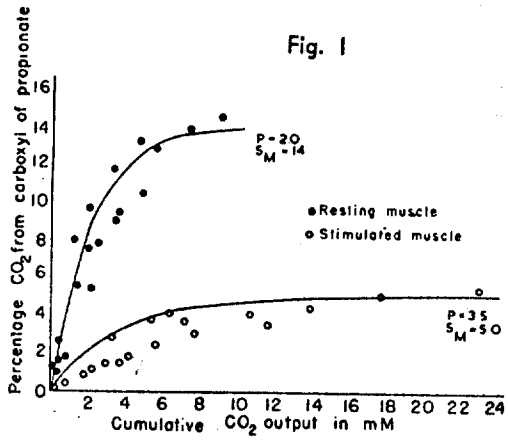
Percentage of Respiratory CO_2 From Administered Propionate in Resting Muscle. Figure 1 shows that labeled CO_2 was collected following the administration of carboxyl-labeled propionate at calculated initial concentrations of 16–22 mM/l. The specific activity of the CO_2 increased with time until in the final collection period, up to 14% of the CO_2 was derived from the carboxyl carbon of the propionate. For reasons to be given below, cumulative CO_2 output has been used as the abscissa of figure 1 instead of time. When the muscle was omitted from the system, the CO_2 collected, consisting no doubt mainly of preformed CO_2 not removed by preliminary aeration, did not contain significant excess C^{13}.

The progressive increase in the C^{13} concentration in the respiratory CO_2 collected with the muscle present might be due to *a*) dilution of metabolic CO_2 by preformed CO_2, *b*) permeation delay, or *c*) metabolic delay, or some combination of these factors. An attempt to evaluate the dilution or 'wash-out' effect was made as follows. Gas analysis of the blood indicated that 2–3.5 mM of CO_2 was present in the blood-muscle system after preliminary aeration and at the time the propionate was administered. If it is assumed that the metabolic CO_2 is mixed uniformly with the CO_2 already present and that the CO_2 collected is representative of this mixture at any given instant, the specific activity of the respiratory CO_2 would then follow the relationship:

$$S = S_M(1 - e^{-R/P})$$

where S is the specific activity of the collected CO_2, S_M is the specific activity of the metabolic CO_2 (assumed to be constant during the experiment), R is the total millimoles of CO_2 produced, and P is the millimoles of CO_2 present in the system. The theoretical line for the non-stimulated muscles has been plotted in figure 1 with S_M taken equal to the highest observed value, i.e., 14%, and P = 2 mM. The agreement between the theoretical and observed values is sufficiently close to suggest that the major factor accounting for the rise in specific activity of the respiratory CO_2 with time is indeed the dilution or 'wash-out' effect, and that the specific activity of the CO_2 arising from cell metabolism is relatively constant during the experiment. In other words, if the amount of $C^{13}O_2$ calculated to be present in the system at the end of the experiment were added to the collected $C^{13}O_2$, an average value for the percentage of CO_2 derived from the carboxyl of propionate over the entire experimental time would be approximately equal to the maximum percentage observed.

On the assumption that all three carbon atoms of propionate are converted to CO_2 at



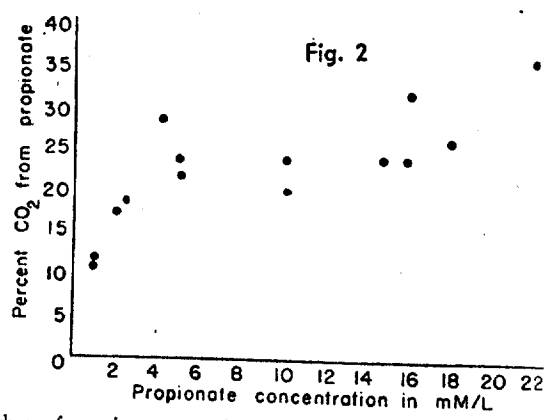


FIG. 1. Percentage of CO_2 derived from carboxyl carbon of propionate as a function of cumulative CO_2 output. Lines are theoretical ones (see text).

FIG. 2. Relationship between percentage of CO_2 from administered propionate and calculated initial propionate concentration. Comparison made at 3 mM of cumulative CO_2 output (see text).

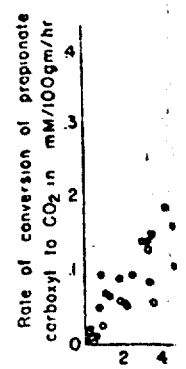


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Fig. 2

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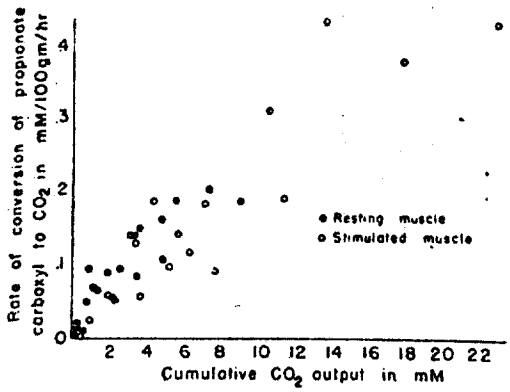


FIG. 3. Rate of conversion of propionate to CO_2 as a function of total CO_2 output.

the same rate, the values computed for the percentage of the respiratory CO_2 derived from the administered propionate would be triple those given for the carboxyl carbon, and would reach more than 40% in resting muscle. However, the fate of the carboxyl carbon may not be representative of that of the alpha and beta carbons of propionate. For example, propionate might be converted to a symmetrical form of a C_4 dicarboxylic acid which undergoes rapid decarboxylation. Under these conditions the labeling of the carboxyl carbon of propionate would be lost without simultaneous or as extensive conversion of the alpha and beta carbons to CO_2. It has been reported that after the intravenous injection of labeled propionate into the intact dairy cow, the carboxyl carbon appears in the respiratory CO_2 2–3 times as rapidly as the alpha carbon (3). However, in the rat after oral administration of the compound, the recovery of labeled carbon in the CO_2 was roughly equal for all three carbon atoms (4).

Figure 2 shows the effects of reducing the initial propionate concentration on the contribution of the carboxyl carbon of this substrate of CO_2. For purposes of comparison, the values have been plotted for the percentage of the CO_2 derived from the labeled compound at a total CO_2 output of 3 mM. In several instances the experiment was not run quite long enough to yield a point at 3 mM of CO_2, in which cases, the plotted value was obtained by a slight extrapolation. Decreasing the initial substrate concentration to 4 mM/l. had a relatively minor effect, but at 1 mM/l. the value for the specific activity of the CO_2 is reduced by about 50%. From figure 2 the saturating propionate concentration would appear to be of the order of 4 mM/l. It is probably less than this because the isotopic substrate levels diminish with time due to utilization and this complication is more important at the lower substrate levels. Thus in the two experiments at the 1 mM/l. concentration, 32% and 50%, respectively, of the injected C^{13} was accounted for in the collected CO_2 alone. On the other hand, in the five experiments in the 15–22 mM/l. concentration range, only 8% of the administered isotope was recovered in the CO_2. This would represent only a negligible decrease in substrate level.

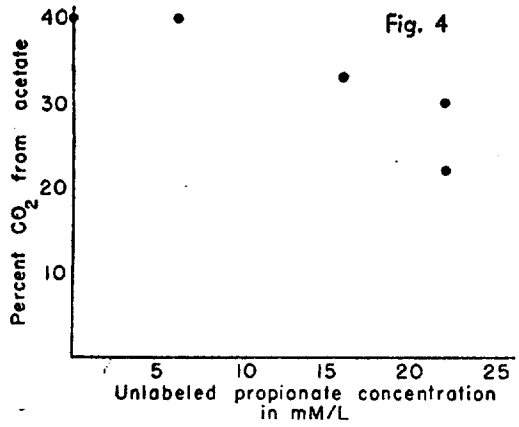
It may be pointed out that even the lowest propionate concentration employed (1 mM/l.) is much higher than the normal one for dog blood, since the total volatile fatty acid level has been reported to be less than 0.5 mM/l. (5, 6) of which only 1–4% is propionic acid (6). It may also be pointed out that no glucose or insulin was added to the perfusion blood.

Percentage of Respiratory CO_2 From Administered Propionate in Repetitively Contracting Muscle. When the muscle was stimulated maximally at a rate of 2 sec. via its nerve, less than 6% of the respiratory CO_2 was derived from the carboxyl carbon of propionate (fig. 1) or 18% from the compound, again on the assumption of equal rates of conversion for all three carbon atoms. This is less than one-half the value observed for resting muscle. The early increase in specific activity of the collected respiratory CO_2 with cumulative CO_2, in the case of the active muscle, did not appear to be as rapid as would be anticipated from the 'wash-out' of preformed CO_2. The theoretical line has in this case been calculated on the assumption that P = 3.5 in order to correct maximally for such 'wash-out.' This result suggests that, in addition to the dilution effect of the latter, permeation or metabolic delay or both are perhaps operating to some extent, and that the C^{13} concentration of the CO_2 released from the muscle cells may well have increased with time. This increase may have been related to progressive depletion of other available substrates by the augmented metabolism.

Rate of Conversion of Administered Propionate to CO_2 in Resting and Contracting Muscle. Figure 3 shows the rate of conversion of the carboxyl carbon of the administered propionate to respiratory CO_2 as a function of cumulative CO_2 output for substrate concentrations of 16–22 mM/l. In the resting muscles the highest rate observed was 0.2 mM/100 gm of muscle/hr. Up to the time when the cumulative CO_2 output was about 10 mM the resting and active muscles yielded rather similar results. One may thus infer that during this initial period propionate did not contribute substantially to the metabolic mixture giving rise to the additional CO_2 associated with repetitive contraction. Rather, the lower specific activity of the respiratory CO_2 of the stimulated preparations as compared with resting ones (fig. 1), is explained by a disproportional increase in production of CO_2 from substrates other than propionate (or those with which propionate may be in isotopic equilibrium). However, with continued repetitive contractions and exposure to the propionate, the rate of conversion of the carboxyl carbon of this compound to CO_2 increased to some 0.4 mM/100 gm/hr., or about double the maximum observed for resting muscles. For resting muscle to show this rate, it would require that 80%–100% of its CO_2 output come from the propionate on the assumption of equal rates of conversion to CO_2 for the three carbon atoms of the latter. Apparently with continued activity propionate approximately doubles its absolute contribution to the metabolic mixture compared to that in the resting state. This is consistent with the observation that in the case of contracting preparations the course of the specific activity of the respiratory CO_2 did not appear to be accounted for exclusively by 'wash-out' of preformed CO_2. However, since the rate of total CO_2 output was increased much more by activity (from an average of 1.2 mM to an average of 5.9 mM/100 gm/hr.) than was the rate of conversion of the carboxyl carbon of propionate to CO_2, the specific activity of the CO_2 during activity does not approach the corresponding value during rest.

This treatment of propionate by contracting as compared to resting muscle differs from that of either administered acetate or lactate, under comparable conditions. In the case of acetate, approximately the same high proportion of the respiratory CO_2 (of the same order as that of propionate in resting muscle) is derived from this substrate during either rest or stimulation. By contrast, in the case of the lactate, the percentage contribution of the carboxyl carbon of lactate to CO_2 actually doubles during contraction despite the severalfold augmentation in total CO_2 output (7).

Conversion of Administered Propionate to Lactate by Muscle. In the control experiments on blood alone, the blood lactate was isotope-free (table 1). When the gastrocnemius was



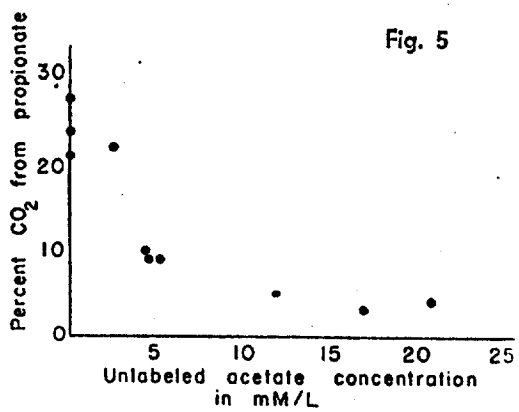


FIG. 4. Influence of concentration of unlabeled propionate on percentage of CO_2 derived from labeled acetate (4.8–6.6 mM/l. initial concentration). Comparison made at 3 mM of cumulative CO_2 output.

FIG. 5. Influence of concentration of unlabeled acetate on percentage of CO_2 from labeled propionate (4–6 mM/l. initial concentration). Comparison made at 3 mM of cumulative CO_2 output.

TABLE 1. DATA

Type of Expt.	Mus Glyco	
	Control Muscle	
Resting	1010	
Resting	1310	
Resting	627	
Stimulated	362	
Stimulated	658	
Stimulated	668	
Stimulated	442	
Blood control		
Blood control		

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Fig. 5

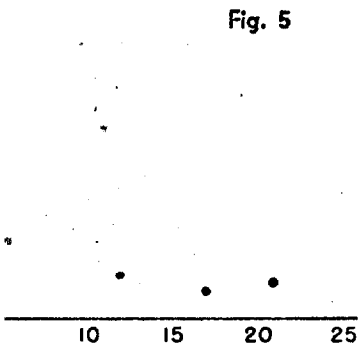

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TABLE 1. DATA ON MUSCLE GLYCOGEN AND BLOOD LACTATE

Type of Expt.	Muscle Glycogen, mg		C^{13} in Blood Lactate		C^{13} in Admin cpd	C^{13} in Final Resp. CO_2
	Control Muscle	Perfused Muscle	Carboxyl Carbon	α,β Carbons		
			atom % excess			
Resting	1010	875	.14	−.01	2.75	1.06
Resting	1310	1294	.12	−.01	2.75	.87
Resting	627	925	.36	.03	2.75	1.20
Stimulated	362	316	.02	.01	2.48	.28
Stimulated	658	468			2.67	.44
Stimulated	668	520	.06	.00	2.67	.41
Stimulated	442	305			2.67	.28
Blood control			.00	.00	1.25	.01
Blood control			.00	.00	2.75	.01

included in the system, the degradation fractions containing the alpha and beta carbons of lactate showed a maximum C^{13} of only .03 atom % excess, whereas the fractions containing the carboxyl carbon varied in C^{13} content from .02 to .36 atom % excess. The latter value is still only 4% of the concentration in the carboxyl carbon of the administered propionate. Although the blood lactate was not rigorously purified, it appears reasonably certain that the muscle formed carboxyl-labeled lactate after the administration of carboxyl-labeled propionate. This conversion would be anticipated, by CO_2 fixation or otherwise, from current concepts of propionate metabolism in tissues (4, 8–12). Comparison with the values for the C^{13} of the terminal respiratory CO_2 (table 1) reveals that higher specific activities in the carboxyl of the blood lactate were associated with higher specific activities in the respiratory CO_2, but that the C^{13} abundance in the CO_2 was always much greater than in the carboxyl carbon of the lactate. This suggests that lactate may not be in the direct pathway from propionate to CO_2.

Failure to Demonstrate Conversion of Administered Propionate Carbon to Glycogen. The glycogen from each of seven muscles (3 resting, 4 stimulated) was isolated and analyzed for C^{13}. Included are experiments in which the glycogen content of the perfused muscle increased, remained approximately constant, or decreased as judged by comparison with the glycogen content of the unperfused muscle of the other leg (table 1). Since the C^{13} abundance in the administered propionate was 2.48–2.75 atom % excess, a value of .02–.03 atom % excess would have occurred in the isolated glycogen if 1% of the latter had been formed from the administered propionate. In each instance the glycogen was isotopically normal.

Competition Between Administered Acetate and Propionate for Conversion to CO_2 in Resting Muscle. As much as 20% of the respiratory CO_2 of resting muscle can be derived from the carboxyl carbon of acetate or 40% from the compound on the assumption of equal rates of metabolism to CO_2 for the two carbon atoms (fig. 4). It was therefore of interest to study muscles to which acetate and propionate were administered simultaneously. In some experiments the acetate was labeled, in others the propionate. It was found that the individual contributions of the two compounds to respiratory CO_2 were less than additive. Moreover, acetate was a much more potent inhibitor of propionate in this respect than vice versa. For example (figs. 4 and 5), at 3 mM of cumulative CO_2 production and at initial substrate concentrations of 5–7 mM/l., which are saturating levels, or nearly so, for each compound, 25% of the respiratory CO_2 was derived from propionate and 40% from acetate, when the compounds were given individually. When administered together, acetate still contributed 40%, but the propionate only about 10%, for a total of 50% from both compounds, instead of 65%. When the acetate level was raised to 17 mM/l., only 3% of the CO_2 was derived from the propionate. In the reverse situation (acetate 5.9 mM/l., propionate 16 mM/l.) the value for acetate was 34%. It should be added, however, that at the lower concentrations of unlabeled acetate there was a tendency for the inhibitory effect on propionate metabolism to diminish with time. This may have been related to metabolic removal of the acetate.

There are many other possible explanations for the finding that acetate can compete successfully with propionate for conversion to CO_2, but an obvious one is that common components of the respective activating systems have a greater affinity for acetate than for

propionate. The failure of the rate of conversion of propionate to CO_2 to increase proportionately with the rate of total CO_2 production during stimulation may also be related to this competitive phenomenon, in that the onset of activity may somehow make available substrates which, like acetate, displace propionate.

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SYNTHESIS OF MILK FAT IN THE BOVINE MAMMARY GLAND [1]

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SUMMARY

Milk fat synthesis was studied in normal lactating dairy cows following the intramammary infusion of C^{14}-labeled glycerol, glucose, acetate, propionate, and butyrate. The metabolite under investigation was infused into one quarter (or half) of the udder and a comparison was made of the C^{14} content of milk fat glycerol obtained from the infused and uninfused quarters. Our results indicate that glycerol is synthesized in the mammary gland from glucose but not from acetate, propionate, or butyrate. Further, we have shown that the newly formed glycerol is incorporated into milk fat. This strongly implies that one pathway of milk fat formation involves its *de novo* synthesis in the mammary gland from pools of free fatty acids and glycerol.

Milk fat consists almost entirely of mixed triglyceride in which glycerol is esterified with various combinations of at least 14 different fatty acids. It is now generally accepted that milk fat glycerol and the individual fatty acids themselves are synthesized from widely different precursors in the ruminant body, but there is little evidence to suggest where the fatty acids are esterified with glycerol to form the ultimate product, milk fat. Further, McCarthy *et al.* (8) recently proposed that two mechanisms might be involved in milk fat synthesis: alteration of pre-existing triglyceride molecules and supplemental synthesis (from nonlipid precursors).

Large quantities of glyceride are absorbed from the blood into mammary tissue (2, 7, 11). This suggests that milk fat might be formed by a fatty acid rearrangement or substitution on these preformed glyceride molecules. Indeed, a recent paper by Kumar and Lalka (4) provided direct evidence of this mechanism.

In contrast, milk fat might be assembled within the mammary gland, using glycerol and free fatty acids as building blocks. The presence of short-chain fatty acids in the mammary gland is now well established [see recent review by Luick (5)]. Intramammary synthesis of milk fat glycerol was denied by Glascock (1); however, in 1958 Wood and coworkers reported strong evidence for it (14).

This paper provides new evidence that glycerol is normally synthesized from glucose in bovine mammary tissue. We have also established that the newly formed glycerol is incorporated into milk fat. Our results almost certainly mean that milk fat is synthesized, at least in part, in the mammary gland from pools of free fatty acids and glycerol.

EXPERIMENTAL PROCEDURE

Isotope. The C^{14}-labeled substances under investigation were dissolved in 50 ml. of sterile isotonic saline and infused through a teat cannula into the

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TABLE 1

Experimental details relevant to the isotope administered, site of infusion, and milk yield during the various trials

Substance infused	Millicuries Infused	Site of infusion	Milk yield (grams)			
			Milk 1		Milk 2	
			Infused quarter	Uninfused quarter	Infused quarter	Uninfused quarter
2-C^{14} glycerol	1.92	Right front	387	1,600	597	2,661
2-C^{14} glycerol	1.50	Right half	863	740	713	799
UC^{14} glucose	2.47	Right front	320	1,820	590	2,770
UC^{14} glucose	2.16	Right rear	630	1,980	856	1,835
2-C^{14} propionate	1.65	Left rear	454	590	447	729
2-C^{14} butyrate	1.37	Right front	660	1,660	460	723
2-C^{14} butyrate	1.24	Right front	306	1,550	318	1,640
2-C^{14} acetate	2.46	Right front	250	690	286	581
2-C^{14} acetate	2.77	Right front	410	1,950	360	2,360

udders of lactating dairy cows. Table 1 shows data pertaining to the metabolite studied, site of infusion, and milk yield of the infused and uninfused quarters.

Cows. The cows were milked out before infusion of the isotope and, unless otherwise noted, at 3, 12, and 24 hr. thereafter. Each milk sample was stored for 24 hr. at 4° C. to allow the cream to separate from the skimmilk. Milk fat was extracted from the cream by the Roese-Gottlieb technique. Glycerol was isolated from milk fat as the tribenzoate derivative with techniques described by Luick and Kleiber (6).

Degradation of glycerol. The distribution of C^{14} on the glycerol skeleton was studied according to a method published by Kritchevsky and Abraham (3). C-1 and C-3 atoms were obtained as the dimedon derivative following saponification with 0.6 *N* alcoholic KOH and oxidation of the glycerol moiety with $NaIO_4$ (12). Oxidation of a second glycerol sample with $Pb(OAc)_4$ yielded C-2 as CO_2 and C-1 and C-3 as formaldehyde.

Radioassay. The organic compounds were oxidized to CO_2 in a microfurnace, and $BaCO_3$ planchettes of infinite thickness were prepared for counting under a micromil G.M. detector. The results are expressed as net counts per minute and, except for the data in Table 3, have not been corrected for the eightfold dilution effect of tribenzoate C, even though this correction would be justified for samples containing high C^{14} content. Rather, the actual counting rates are listed, to prevent attaching undue significance to samples of low activity. These counting rates, when corrected for dilution, may be expressed as specific activity according to the formula: c/m × 0.047 = μc. C^{14}/gm at C.

RESULTS AND DISCUSSION

The criterion by which we decide whether a specific metabolite is used by mammary tissue for the synthesis of a given product (in this case, glycerol) rests on a comparison of the relative counting rates of that product isolated from the infused and uninfused quarters. If the metabolite is not utilized directly by mammary tissue, it would have to be taken into the general circulation and returned to the udder in a usable form before it could appear in that

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TABLE 2

C^{14} in milk fat glycerol following intramammary infusion of various metabolites

Isotope infused	Milk No.	Time after infusion	C^{14} in glycerol tribenzoate: Infused quarter c/m	C^{14} in glycerol tribenzoate: Uninfused quarters c/m	Ratio infused/uninfused
		(hr.)			
Glycerol 2-C^{14}	1	3.5	455	19	24/1
(Trial I)	2	10	355	41	9/1
	3	24	63	9	7/1
Glycerol 2-C^{14}	1	3.2	37	6	6/1
(Trial II)	2	6.0	100	36	3/1
	3	12	120	53	2/1
Glucose U-C^{14}	1	3.3	161	8	20/1
(Trial VII)	2	8.4	60	55	1/1
	3	24	42	30	1/1
Glucose U-C^{14}	1	3.3	27	9	3/1
(Trial VIII)	2	9.7	135	103	1/1
	3	24.7	58	46	1/1
Butyrate 2-C^{14}	1	3.0	3	1	3/1
(Trial VIII)	2	9.3	13	11	1/1
Butyrate 2-C^{14}	1	3.2	9	4	2/1
(Trial IX)	2	8.5	28	18	2/1
Propionate 2-C^{14}	1	4.5	6	2	3/1
(Trial VI)	2	10.3	4	18	1/4
Acetate 2-C^{14}	1	3.2	1	1	1/1
(Trial XVI)	2	8.0	12	9	1/1
Acetate 2-C^{14}	1	3.5	4	1	4/1
(Trial XVII)	2	8.5	19	12	2/1

milk product. In this case, the C^{14} content in the product taken from the uninfused quarter would be equal to that of the infused quarter. The validity of this criterion depends chiefly on the assumption that the infused metabolite can not pass from one quarter to another without first entering the general circulation. We think this assumption is justified, since there is little evidence to suggest a circulatory interchange between the various quarters (13) and, further, in similar trials with Fe^{59}- and P^{32}-labeled metabolites (Luick *et al.*, unpublished), we found no evidence that substances exchange or diffuse from one quarter to another.

TABLE 3

Distribution of C^{14} in milk fat glycerol 3.5 hr. after the intramammary infusion of 2-C^{14} glycerol (Trial 1). All values have been corrected for the diluting effect of the derivative C

Sample	Counting rate, counts/minute: 2C	C-1 + C-3	C-2
Infused quarter	4,540	220 [a]	13,200 [c]
		218 [b]	6,900 [d]
Uninfused quarter	160	174 [a]	193 [d]
		156 [b]	150 [c]

[a] Dimedon derivative following $NaIO_4$ oxidation.
[b] Formaldehyde fraction following $Pb(OAc)_4$ oxidation.
[c] Theoretical value based on counting rate of 2C and C-1 + C-3 according to the equation: $c/m_{(C-2)} = 3\ c/m_{(2C)} - 2\ c/m_{(C-1\ +\ C-3)}$.
[d] CO_2 fraction following $Pb(OAc)_4$ oxidation. The discrepancy between this value and the theoretical value noted for the infused quarter is presumably due to dilution with C during the oxidation procedure.

Table 2 lists the counting rates of glycerol samples taken from the infused and uninfused quarters following intramammary infusion of C^{14}-labeled glycerol, glucose, butyrate, propionate, and acetate. These metabolites have been listed in order of decreasing maximum count rate of glycerol taken from the infused quarters (and interestingly, decreasing ratio of infused quarter to uninfused quarter). Thus, comparison can be made between the different metabolites, e.g., glucose vs. acetate, in their ability to label glycerol. However, this comparison must be viewed with caution, since we have no information relevant to the extent of absorption of these substances into the udder.

Intramammary fixation of glycerol. The high labeling of milk fat glycerol taken from the quarter infused with 2-C^{14} glycerol is of particular interest, since it indicates that glycerol—if present as such in the mammary gland—may be incorporated into milk fat. In this regard, our results confirm a similar observation by Wood *et al.* (15) following injection of 1,3-C^{13} glycerol into the pudic artery of a lactating cow. In each of our trials, however, the high C^{14} content of glycerol might have occurred fortuitously via processes that did not require a net synthesis of milk fat.

First, it seemed remotely possible that the infused glycerol might have exchanged with the nonradioactive glycerol moiety of milk fat. We tested this possibility by injecting 380 $\mu c.$ of 2-C^{14} glycerol into 1 liter of freshly drawn milk. This sample was then divided into two 500-ml. portions, and milk fat was extracted from each portion by techniques similar to those used in the cow trials. Glycerol was isolated from the milk fat as tribenzoate and radioassayed for C^{14} content. These derivatives gave less than three net counts per minute above background. We concluded that glyceride glycerol does not exchange in vitro with nonesterified glycerol.

Secondly, we were concerned that the infused C^{14}-labeled glycerol might have been degraded into metabolically active fragments that could, in turn, label glycerol through exchange relations. This degradation and resynthesis could occur within mammary tissue or through microbial activity during sample preparation. Regardless of cause, however, it is almost certain that the final result would be a significant translocation of the C^{14} label from the C-2 position to the C-1 and C-3 positions. Accordingly, a study was made of the distribution of C^{14} in milk fat glycerol (Trial 1). The results are in Table 3, where it may be seen that most of the C^{14} in glycerol from the infused quarter was on the C-2 position. This labeling pattern is almost identical with that of the infused 2-C^{14} glycerol. We, therefore, conclude that the high radioactivity found in milk fat glycerol means that glycerol is normally incorporated into milk fat in the mammary gland provided a supply of glycerol is available for this purpose.[2]

[2] Small but significant amounts of C^{14} on positions C-1 and C-3 do not necessarily mean that the infused glycerol was catabolized in mammary tissue. In fact, the even distribution of C^{14} on the C skeleton of glycerol taken from the uninfused quarter, and the similarity in count rate between the C atoms and the C-1 and C-3 positions from the infused side, indicate considerable catabolism of the infused material subsequent to its absorption from the mammary gland.

Intramammary synthesis of milk fat glycerol. Table 2 also lists the relative counting rates of glycerol samples obtained after intramammary infusion of glucose, acetate, propionate, and butyrate. These data show that in the lactating cow, only glucose leads to a significant labeling of glycerol, confirming an earlier report by Wood and coworkers (14), who injected 2, 6-C^{14} glucose into surgically prepared cows. Similarly, it refutes Glascock's conclusion, following a review of udder perfusion and mammary-slice experiments that the ". . . mammary gland is not the site of production of glycerol." The failure to demonstrate glycerol synthesis in the perfused udder (and in the mammary slice) seems understandable in light of recent criticisms of this technique (10, 14).

A considerable difference is noted in the extent of C^{14} incorporation into milk fat glycerol in Trials Glucose VII and Glucose VIII. This is particularly evident in the first milk samples taken from the infused quarters. The relatively low C^{14} content of milk fat glycerol, Trial Glucose VIII, might have been caused by a failure to completely milk-out the quarter prior to the infusion. This situation would lead to a dilution of the newly synthesized milk fat and presumably could have been prevented through the use of the milk let-down hormone, oxytocin. However, we have deliberately avoided the use of oxytocin, in order to maintain the one great advantage of our experimental technique—namely, the use of intact, lactating dairy cows under quasi-normal conditions. Perhaps equally important, Shaw and Petersen (11) have reported that oxytocin interferes with milk fat synthesis by decreasing the passage of plasma lipids into mammary tissue.

The data in Table 2 also show that the second milk fat glycerol samples taken from the infused quarters (Trials Glycerol II and Glucose VIII) contain more C^{14} than similar samples taken from the first milk. This delay in appearance of peak activity in milk fat invariably occurs following intravenous injection of C^{14}-labeled metabolites and presumably reflects delays in the synthesis and secretion of newly formed milk fat. This delay occurs in the same trials where we observe the least difference in count rate between milk fat glycerol samples taken from the infused and uninfused quarters. This suggests a diminished rate of milk fat synthesis in the mammary gland during the first 3 hr. of the trials as an alternative explanation for the inconsistencies noted in the Glucose and Glycerol trials.

Origin of milk fat. The establishment of mammary synthesis of milk fat glycerol from glucose is of particular importance, since large quantities of glucose are absorbed into the udder from the blood during lactation (2, 9). Further, Luick and Kleiber (6) recently reported that at least 70% of milk fat glycerol is synthesized from blood glucose. They postulated that this synthesis might proceed by one (or both) of two pathways: (a) synthesis of glycerol from glucose in mammary tissues, or (b) synthesis of plasma lipid glycerol, presumably in the liver, and its subsequent absorption as glyceride glycerol into the udder. The results of our experiments and the report by Wood *et al.* (14) provide strong evidence for intramammary synthesis of glycerol from glucose and for incorporation of the newly formed glycerol into milk fat. This means that milk fat is synthesized *de novo* within the mammary gland from pools of free fatty acids and glycerol. It does not exclude, however, the simultaneous formation of milk fat from plasma glyceride via mechanisms involving fatty acid exchange, substitution, or rearrangement within the mammary gland. Indeed, the recent observations of McCarthy *et al.* (8) may be readily explained if both mechanisms contribute to milk fat synthesis in the lactating cow.

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notes on methodology

Determination of volatile free fatty acids of human blood

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SUMMARY A simple and accurate method for the extraction and quantitative estimation of individual, volatile free fatty acids of human blood is described. Values in normal serum, plasma, and red cells are given. Besides acetic and propionic, normal and isobutyric and isovaleric acids have been found.

SUPPLEMENTARY KEY WORDS extraction · gas–liquid chromatography · aqueous solutions · polymer beads · butyric · valeric

THE NUTRITIONAL importance of volatile fatty acids (VFA) in the ruminant has led to many investigations concerning these compounds of low molecular weight, including their estimation in the blood (1–4). However, the significance of these short-chain acids in the blood of nonruminants, especially man, has not been properly studied and methods for their extraction, identification, and quantification have not been standardized.

Various procedures have been described in the literature for obtaining VFA concentrates from ruminant blood. Erwin, Marco, and Emery (5) lyophilized the deproteinized blood filtrate after neutralization with alkali and extracted the VFA with acidified ether or methanol. Ramsey (6) found that the deproteinized filtrate contained considerable amounts of substances other than the organic acids and deemed it necessary to remove them before quantifying the VFA by gas–liquid chromatography (GLC). Scarisbrick (7) recommended estimation of blood VFA by steam distillation of whole blood in the presence of potassium oxalate and oxalic acid (pH 4.0), but recoveries and values for blood VFA were not given.

McClendon (8) steam-distilled deproteinized human blood in the presence of phosphoric acid and reported a titer of 0.0003 N for total blood VFA. Annison (4) steam-distilled deproteinized blood filtrates at pH 3.0 and reported 0.28–0.3 mmole of total VFA per liter. Formic, acetic, and propionic acids constituted 23–34, 64–77, and 0–4 moles%, respectively. No other VFA was identified. Friedemann (9) conducted steam distillation of blood in the presence of magnesium sulfate, since high concentrations of $MgSO_4$ were shown to increase the rate of distillation of VFA (10). McAnnally (11) found that the VFA content of a constant volume of distillate obtained by the Friedemann procedure increased with the time taken for distillation, because of the decomposition of labile substances.

Recently, Tanaka, Budd, Efron, and Isselbacher (12) removed total free fatty acids from a chloroform-methanol extract of the total lipids of serum by means of 0.1 N NaOH. The alkaline extract was acidified with phosphoric acid and steam-distilled to obtain VFA.

The methods of estimation of VFA described in the literature have been just as varied as the methods described above for their extraction from blood. Earlier methods of partition and adsorption chromatography used to separate individual short-chain fatty acids (13, 14) have been supplanted by GLC. Because of the many difficulties encountered during GLC analyses of aqueous solutions of VFA, such as tailing and incomplete separations, methods were devised to replace the aqueous medium with organic solvents (15). The use of the flame-ionization detector, which is insensitive to water, has obviated these difficulties and aqueous solutions of VFA can now be analyzed directly.

This paper describes a simple procedure for the extraction of circulating VFA from human serum in good yields and the quantification of the normal and iso acids by GLC on polymer beads. The distribution of the various VFA between the red cells and plasma of normal human blood is also reported.

Materials. All solvents used for extraction purposes were AR grade and distilled. Glass-distilled water was used wherever necessary.

Venous blood from fasting humans was collected in Vacutainer tubes containing oxalate and fluoride. Plasma was separated from the red cells by centrifugation at 750 *g* for 30 min. The red cells, free from "buffy coat," were washed three times with cold 0.15 M saline. To obtain serum, we collected blood in ordinary glass tubes, which we kept at 37°C for 1 hr and then centrifuged.

Extraction of VFA. A satisfactory recovery of VFA from blood is an important prerequisite to their subsequent separation and quantification by GLC. The following method gave a higher yield of VFA than the procedures described by Friedemann (9) and Scarisbrick (7), and in addition revealed hitherto unreported VFA. The procedure used to prepare VFA concentrate from

Abbreviations: VFA, volatile fatty acid; GLC, gas–liquid chromatography.

TABLE 1 Determination of Acids in Two Standard Mixtures of Acids

	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	n-Valeric
Mixture 1						
Amount present, μg	40.9	39.2	37.6	37.2	37.3	36.1
Amount estimated (mean ± SD, n = 6)	38.8 ± 1.7	38.9 ± 0.9	37.9 ± 1.0	37.1 ± 1.3	38.1 ± 0.8	36.1
% of expected	94.9	99.3	100.7	99.7	102.2	100.0
*Mixture 2**						
Amount present, μg	82.2	8.63	8.24	1.31	4.12	30.0
Amount estimated (mean ± SD, n = 6)	79.6 ± 0.7	8.46 ± 0.08	8.01 ± 0.28	1.28 ± 0.03	4.06 ± 0.05	30.0
% of expected	96.9	98.0	97.2	97.7	98.3	100.0

* Contained amounts of the acids normally found in 5 ml of plasma.

TABLE 2 Recovery of Pure Acids Added to Serum

	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	n-Valeric
Acid added, μg	40.9	39.2	37.6	37.2	37.3	36.1
Acid recovered, μg	41.3	39.3	37.7	38.5	36.7	36.1
Recovery %	100.9	100.2	100.2	103.0	98.4	100.0
Acid added, μg	32.0	31.0	29.0	29.0	29.0	28.0
Acid recovered, μg	32.2	30.3	28.2	29.7	28.6	28.0
Recovery %	100.7	97.7	97.4	102.4	99.2	100.0

plasma described below is applicable also to serum and red cell fractions.

Plasma (5 ml) was extracted with 20 volumes of chloroform–methanol 2:1 in the presence of 0.2 volume of 1.0 N H_2SO_4 in a Servall Omnimixer and filtered. The residue was washed thrice with 10 ml portions of the same solvent mixture. The filtrates were combined and mixed with a volume of water equal to 0.2 the total volume of the chloroform–methanol extract, and the two phases were allowed to separate. After the interface had become sharply defined, the lower (chloroform) phase was removed and extracted with 50 ml of methanol–water 1:1 that had been equilibrated with 10 ml of chloroform. The aqueous-methanolic fractions were combined, neutralized with 1 N KOH to pH 8.0–9.0, and evaporated to dryness. The residue was acidified with 25% aqueous metaphosphoric acid to pH 3.0, 5.0 g of $MgSO_4$ was added, and the mixture was steam-distilled in a micro-Kjeldahl distillation unit (Labconco, Kansas City, Mo.). At this pH pyruvic, lactic, and β-hydroxybutyric acids, at concentrations normally encountered in blood, are not distilled off (4) and hence do not interfere with the determinations of VFA. 200 ml of distillate was collected in 0.3 ml of 1 N NaOH (pH 9.0–10.0) and evaporated to dryness in vacuo. The residue was made up to a known volume with 25% aqueous metaphosphoric acid and the VFA were analyzed by GLC.

GLC. The aqueous phosphoric acid solutions of the VFA concentrates were chromatographed on a Beckman GC-4 dual-column instrument equipped with flame-ionization detectors. The aluminum columns were 8 ft × 1/16 inch (I.D.) and were packed with 150–200 mesh Porapak Q [beads of ethylvinylbenzene–divinylbenzene polymer (Waters Associates, Framingham, Mass.)] coated with 4% phosphoric acid. Details of quantitative analysis of VFA, including column preparation, separation, and area-response factors for individual VFA with respect to *n*-valeric acid have been described previously (16). Since *n*-valeric acid was not found in blood, all quantitative analyses were performed with *n*-valeric acid as the internal standard.

Results. The over-all accuracy of the extraction and separation of the acids was tested with standard solutions of the mixed acids. Table 1 shows the results obtained in the analysis of two synthetic mixtures containing different concentrations of the acids. Mixture 1 contained roughly equal amounts of the acids and mixture 2 was made up to approximate the amounts of these acids normally found in 5 ml of plasma. Values obtained were close to 100% of expected for all the acids studied.

Table 2 shows recoveries for pure acids added to serum. Two mixtures containing different amounts of the acids were used; recovery ranged form 97 to 102%.

We checked the reliability of the method for blood VFA analysis by performing six analyses on a pooled sample of human serum (Table 3).

The mean concentrations of five VFA present in the

TABLE 3 Reproducibility of VFA Analysis of Human Serum

Acid	Concentration*
	μg/100 ml
Acetic	1150 ± 16
Propionic	40.5 ± 1.41
Isobutyric	31.5 ± 0.93
n-Butyric	7.9 ± 0.17
Isovaleric	61.0 ± 1.27
Total	1290 ± 14.5

* Mean ± SD (n = 6).

TABLE 4 VFA Content of Normal Human Serum, Red Cells, and Plasma

Acid	Serum	Plasma	Red Cells
	μg/100 ml of fluid or packed cells		
Acetic	1170 ± 136*	1640 ± 378	1400 ± 263
Propionic	42.7 ± 8.9	163 ± 25	188 ± 28
Isobutyric	30.7 ± 7.9	143 ± 30	96 ± 21
n-Butyric	9.7 ± 5.5	25 ± 5	16 ± 4
Isovaleric	58.3 ± 35	194 ± 80	0
Total	1320 ± 170	2170 ± 472	1700 ± 258

* Mean ± SD (n = 6 subjects).

serum of six normal subjects selected at random and the distribution of blood VFA between plasma and red cells of the same subjects are shown in Table 4. Acetic acid constitutes 89% of the total VFA in serum and isovaleric and propionic acids, 4.4 and 3.2%, respectively. Isobutyric and butyric acids are also present in minor amounts. A significant amount of VFA is present in the washed red cells. Acetic and propionic acids of blood are approximately equally distributed between the plasma and the red cells (hematocrits were 45–47.5%). The plasma contains more isobutyric and butyric acids than the red cells. All the isovaleric acid is present in the plasma and none in the red cells. The total VFA content of serum is less than that of either plasma or red cells. Presumably some VFA is adsorbed onto the clot and not completely released in the serum.

The washings obtained during the preparation of red cells were saved and analyzed. Acetic and propionic acids were present in the washings to the extent of 15% of the amounts in the washed red cells. However, it was not possible to conclude whether these acids were derived from the surface of the cells or by transport through the cell membrane. No isobutyric or butyric acid was detected in the washings.

Discussion. The determination of VFA in blood entails essentially two steps: (*a*) extraction and concentration; and (*b*) separation by GLC and quantification. It is imperative that a suitable method for extraction and preparation of a VFA concentrate be employed, not only to obtain the maximum yield but also to permit proper resolution during GLC. Direct injection of as much as 10 μl of serum into the inlet system of the gas chromatograph failed to yield any peaks corresponding to VFA. Direct steam distillation of acidified plasma yielded much less VFA than the steam distillation of the aqueous-methanolic extract. GLC of the extract without steam distillation also yielded much less VFA. It is reasonable to assume, from the above results, that the VFA is bound to a protein or some other constituents of blood and not readily released. A combination of extraction, partition, and steam distillation seems to yield the maximum amount of VFA, since no VFA was detectable in the residue after distillation or in the chloroform phase. In addition, the procedures adopted here yield a VFA concentrate devoid of other substances which interfere with their subsequent determination by GLC. To test the reliability and reproducibility of the method, we have analyzed pooled frozen human plasma at regular intervals. Statistically indistinguishable results were obtained; storage of plasma had no effect on the VFA values.

Many investigators have resorted to the use of methyl esters and butyl esters rather than the free acids for separation by GLC (17–19) because of the troublesome tailing of the free acids on the GLC columns commonly used. The successful separation of aqueous solutions of the free acids on Porapak Q has obviated many of the chores involved in dehydrating aqueous media and esterification reactions.

VFA were identified by comparison of their retention times with those of standards. However, isovaleric and α-methyl butyric acids did not differ in retention time sufficiently to allow them to be separated and unequivocally identified by GLC. It is hoped that this difficulty can be resolved by the use of combined GLC-mass spectrometry.

Although formic acid is present in blood (4) and appears in the steam distillate, it was not detected by the flame-ionization detector. The extraction procedure described afforded a fortuitous and selective separation of the C_2–C_5 short-chain acids from the remaining free fatty acids. We established this by using standard mixtures containing C_2–C_{10} acids; details of these results will be reported later.

Annison (4) did not report the presence of any VFA with longer chains than propionic acid in human blood. The present work has shown that isobutyric and isovaleric acids are also present in significant amounts. n-Butyric acid is also present in small but detectable amounts, but we did not detect n-valeric acid. This is consistent with the observations of Gerson, Hawke, and Shorland (20), who showed that sodium valerate was degraded in vivo to acetate and propionate when administered to cows intravenously. A similar degrada-

tive phenomenon might account for its absence from human blood.

A detailed study of the blood VFA levels in the presence of liver disease is underway in these laboratories.

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Simple liquid–liquid partition system for isolation of labeled oleic acid from mixtures with glycerides

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SUMMARY Oleic acid has a partition coefficient, upper phase/lower phase, of 1.9 (22°C) in the liquid–liquid partition system described herein. Tri-, di-, and monoolein are found almost exclusively in the lower (organic) phase. Oleic acid can be quantitatively removed from mixtures of triglyceride and partial glycerides by means of this partition system under conditions resembling those in a lipase assay.

SUPPLEMENTARY KEY WORDS triolein · diolein · monoolein

LIPASE ACTIVITY has been most commonly assayed by titration of fatty acids produced from hydrolysis of a triglyceride emulsion. The use of radioactive triglycerides greatly increases the sensitivity of the assay but isolation of the fatty acids from the glycerides is time-consuming. To overcome this disadvantage, we have developed a procedure that isolates fatty acids from the glycerides in a one-step liquid–liquid distribution. The radiolabeled fatty acids are then determined directly by liquid scintillation counting.

Materials. Oleic acid-9,10-^{3}H (The Radiochemical Centre, Amersham, Bucks., England) was purified (1) to a radiopurity of 99.5% as shown by thin-layer chromatography (2). Chemical purity was more than 99% by gas–liquid chromatography (3). Oleoyl chloride (The Hormel Institute, Austin, Minn., 99.5% pure) and tri-, di-, and monoolein (Hormel Institute, 99% pure) were used without further purification. Phosphatidyl-U-^{14}C choline (Applied Science Laboratories Inc., State College, Pa., 98% pure) was used without purification. Sodium taurodeoxycholate was obtained from Maybridge Chemical Co., Tintagel, Cornwall, England, with a stated purity of 97.5%. Bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill., Fraction V) was treated with charcoal to remove fatty acids (4). The "potassium carbonate buffer" used in the equilibrations was a potassium carbonate–potassium borate–potassium hydroxide buffer, pH 10.0, 0.05 M (Standard Buffer Solution, Fischer Scientific Co., Fair Lawn, N.J.). All other substances were reagent grade and all solvents were redistilled. All chloroform contained 0.5% (v/v) methanol.

Tri-, di-, and monooleins, ^{3}H-labeled in the oleic acid portion(s), were synthesized from ^{3}H-oleoyl chloride and glycerol according to standard procedures (5, 6). The glycerides were purified by column chromatography on Florisil (7) (Fisher Scientific Co.) to a radiopurity of more than 99.8% (triolein) and more than 99.5% (di- and monoolein). The radiopurity was frequently checked by thin-layer chromatography. Even when

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(2-(5-phenyloxazolyl))benzene.

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Probable role of propionate and butyrate in control of insulin secretion in sheep[1]

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MANNS, J. G., J. M. BODA, AND R. F. WILLES. *Probable role of propionate and butyrate in control of insulin secretion in sheep.* Am. J. Physiol. 212(4): 756–764. 1967.—A chronic surgical preparation was developed which allowed enrichment of pancreatic arterial blood in experiments performed post-operatively in unanesthetized sheep. Experiments with five adult sheep demonstrated that butyrate stimulated insulin secretion through a direct effect on the pancreas which was not due to associated hyperglycemia or hyperketonemia. Butyrate and propionate at rates of infusion within the physiological range stimulated insulin secretion. Whether propionate affected the pancreas directly was not determined. These results indicate that the liver may indirectly control pancreatic islet function in sheep, because extraction of butyrate and propionate from portal blood by the liver governs the concentrations of these compounds in blood reaching the pancreas.

volatile fatty acids; vascular anatomy of ovine pancreas; chronic catheterization of anterior mesenteric artery; insulin immunoassay in sheep; pancreatic infusion in vivo

THE PRECEDING PAPER (15) demonstrated that butyrate, a major product of rumen fermentation, stimulated insulin secretion in sheep to a greater extent than could be explained by the concomitant hyperglycemia. The physiological importance of this response was not assessed, since blood butyrate elevations much in excess of those occurring normally were undoubtedly produced by the intravenous injections employed.

A chronic surgical preparation was developed which permitted local enrichment of the pancreatic arterial blood. Using this preparation, experiments were performed to determine if butyrate and glucose had a direct effect on the pancreas and if propionate and butyrate played an important physiological role in regulating insulin secretion in sheep.

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MATERIALS AND METHODS

Surgical techniques. The gross vascular anatomy of the ovine pancreas has not been extensively studied. The most authoritative work on this subject (12) indicated that much of the pancreatic arterial supply arose from short branches of the anterior mesenteric artery prior to its bifurcation into the ileocecocolic and intestinal trunks. Drainage was by several vessels emptying into the portal vein. To confirm this work, a dissection was performed on a latex preparation. Our results agreed well with those of Happich (12). Chronic catheterization of a specific pancreatic artery and vein did not seem feasible so an alternative approach was adopted.

Several days before surgery animals were placed on a maintenance ration of alfalfa pellets. The animals then were starved for 2 days and water was withheld 1 day prior to surgery. Promazine (2 ml) was used as a preanesthetic and, to produce spinal anesthesia, 6–8 ml Xylocaine (2% in epinephrine) were administered using a 16-gauge 2.5-inch needle. To facilitate readministration of Xylocaine when necessary, a polyvinyl chloride catheter (1.0 mm o.d.) was threaded through the needle shaft into the subdural space. The needle was then removed leaving the catheter in place. Tincture of Zephiran and Zephiran chloride were used as germicidal agents. An incision about 20 cm long was made at the margin of the last rib on the right side. Blunt dissection of muscles revealed the peritoneum, which was opened and expanded with a retractor. The duodenum and jejunum were located and a large branch of both the intestinal artery and vein chosen. After careful dissection, the artery and vein were freed for 6–8 cm and two loose silk ligatures were placed around each vessel. Catheterizations were accomplished by gently lifting the ligatures, cutting small openings in the vessels, and passing the catheters into the lumens. In the case of the vein, catheters were threaded in 22–24 cm and then gently tied in place. Arterial catheters were advanced 30 cm or more to be sure they were into the aorta. If, as in most cases, two portal catheters were required, an additional vein was dissected free and catheterized. It seemed most satisfactory to place the tips of the catheters in the desired position as soon as they were placed within the vessels,

before the latter had constricted around the catheters thus making manipulation difficult. Once the catheters were accurately positioned, they were tied firmly in place. After completion of the catherization, the area of the anterior mesenteric artery which gives rise to the intestinal trunk and the ileocecocolic trunk was located. This is only 8–10 cm from the dorsal aorta and is overlaid by the ventral lobe of the pancreas. The common mesenteric vein lies parallel and close to the mesenteric artery and is easy to rupture. In some instances, depending on the anatomical realtionships in the individual sheep, visual dissection was not possible. After the artery was isolated from connective tissue and from the mesenteric vein at the area of the bifurcation, a Plexiglas clamp containing an inflatable Foley catheter was placed around the artery. This clamp is shown in Fig. 1. The tube of the Foley catheter (French 12) was passed through the peritoneum along with the arterial and venous catheters. The intravessel catheters were tunneled up under the skin and taken out the top of the back where stopcocks (Becton-Dickinson) were attached and tied to the wool. The Foley catheter was secured to the skin by a single heavy silk suture. Figure 2 shows the preparation diagrammatically. This representation is not drawn to scale and for purposes of clarity the venous system is relocated to the right. It actually lies approximately parallel to and under the arterial vessels. Indwelling catheters were soft polyvinyl chloride material (Alphlex, Alpha Wire Co.), 2.0 mm o.d., and would accept the shaft of a 16-gauge needle. The catheters were flushed at least twice daily with heparinized saline (20 U/ml), and were kept full of heparinized saline at all times.

Postoperatively, animals were given 1 M units penicillin and 1.25 g streptomycin intramuscularly twice daily for 4–5 days (Combiotic, Pfizer and Co.). This treatment was reduced to once daily for an additional period of time and subsequently discontinued unless further treatment was indicated. The animals were allowed free access to alfalfa pellets, alfalfa hay, and rolled barley. It was important that they resumed eating as quickly as possible and no attempt was made to limit feed intake. Those which survived 7–10 days after surgery appeared quite normal and were eating satisfactorily. With such a preparation, it was possible to experiment with an unanesthetized animal which had been allowed adequate time to recover from surgery. Animals were not fasted before experiments.

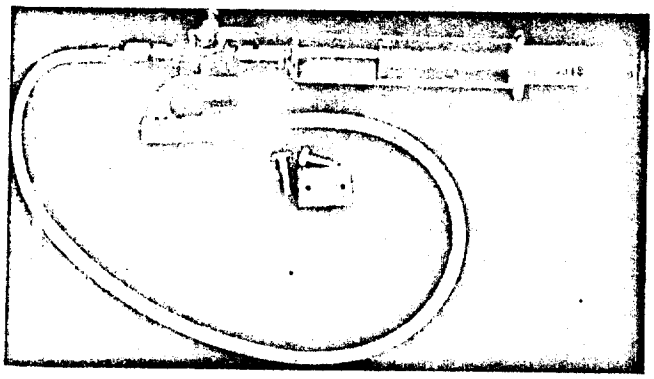

FIG. 1. Plexiglas clamp with inflatable Foley catheter used constrict the anterior mesenteric artery.

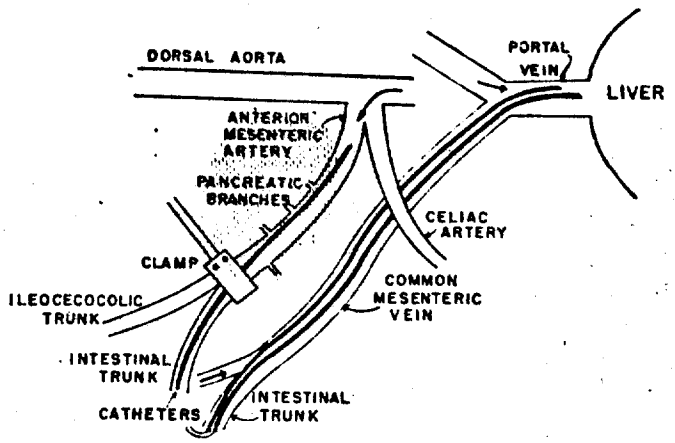

FIG. 2. Diagram depicting placement of indwelling catheters and inflatable clamp. Shaded area indicates approximate position of pancreas; blood flow direction is indicated by arrows. The diagram is not drawn to scale, and for purposes of clarity the venous system is relocated to the right. It actually lies approximately parallel to and under the arterial vessels.

The blood-sampling schedule used in the constant-infusion experiments began with three control samples at 0, 5, and 10 min. After the 10-min sampling, the artery was clamped and the infusion begun. In experiments with the first of the surgically prepared animals (*sheep 75*), samples were taken at 13, 16, 19, and 22 min. At 16 min the clamp was released for 30 sec and then reclamped. At 22 min the infusion was stopped and the clamp released. Additional samples were taken after the end of the infusion period. In experiments with *sheep 0*, *3*, *145*, and *968*, additional samples were obtained at 11 and 12 min. The figures presenting results from individual experiments contain additional details applying to that particular infusion. Continuous infusion was at a rate of 2.0 ml/min in all experiments and was accomplished with either a syringe-type of infusion-withdrawal pump (Harvard Apparatus Co.) or a syringe pump with a pulsating flow (Phipps Bard Co.).

The catheterization technique of Herd and Barger (14), designed for surgically exposed vessels, was adapted for jugular vein catheterization in sheep. Teflon tubing (2.0 mm o.d.) was dipped briefly into a heated paraffin oil-lanolin mixture (about 3:1). The heated portion, held between two needle drivers, was quickly stretched to reduce the diameter so that it would slide into a 16-gauge needle. The resultant piece of tubing was 6–8 inches long with a tapered portion about 2 inches in length. A 16-gauge, 1.5- or 2-inch, thin-wall needle was inserted into the jugular vein against the flow and passed up the vessel for about 1 inch. The needle point was then pushed out through the wall of the vessel and skin so that the tip and the hub were both outside the skin. This was possible because the jugular vein is superficial

in this species. The tapered end of the catheter was threaded through the needle tip; the needle and inserted catheter were then withdrawn. The tapered portion of the catheter was cut off and the remainder drawn back into the vessel until it filled with blood. It was then pushed several inches into the vessel; a stopcock was attached and tied to the wool.

Analytical techniques. Protein-free filtrates of plasma samples for gas-liquid chromatographic determination of individual volatile fatty acids were prepared by adding 0.5 vol of approximately 2 N H_2SO_4 followed by 0.5 vol 20% sodium tungstate. By balancing the concentration of acid with the sodium tungstate a final pH of 3–4 was achieved. Samples were centrifuged for 10 min at 27,000 *g*. The supernatant fluids from 10 to 15 ml plasma were adjusted to pH 3–4 with HCl or KOH and then steam distilled in an apparatus similar to the one described by Fenner and Elliot (10). One hundred milliliters of distillate were collected in a large beaker, an amount previously shown to give quantitative recoveries of acetate, propionate, and butyrate. A drop of phenolphthalein was added to the distillate and 2 ml 0.1 N KOH were added to make the solutions basic. These samples were reduced to dryness by boiling. The beakers were washed with four 2-ml portions of distilled water and the washings combined in a small vial and taken to dryness. Before chromatographic analysis, the salts were converted to acids by taking them up in 0.2 or 0.3 ml of approximately 1 N H_3PO_4. Two to seven microliters of sample were injected into the column of a Barber-Coleman model 15 gas chromatograph equipped with a flame-ionization detector. The carrier gas was helium adjusted to 14 lb pressure. Column and detector temperatures were 145 and 240 C, respectively. The 152 x 0.6-cm glass column was packed with 20% terephthalic acid-Carbowax 20M on Chromosorb W 80/100 mesh. Peak areas of unknowns and standards were calculated by triangulation.

Glucose determination, ketone body analysis, and insulin assays were performed as described previously (15).

RESULTS

Five animals were used to study the effect of butyrate, glucose, and propionate on the pancreas in vivo.

On 27 September 1965, a 50-kg, 5- to 7-year-old ewe (*sheep 75*) was prepared with one portal catheter, one arterial catheter, and the clamp around the artery at its bifurcation. Three days later the experiment resulting in the data shown in Fig. 3 was performed. Infusion of 0.2 mmoles butyrate/min into the clamped anterior mesenteric artery began at 10 and ended at 22 min. Jugular and portal blood samples were taken simultaneously for insulin, glucose, and volatile fatty acid analyses. Analysis of volatile fatty acids was performed on pooled aliquots of the 0-, 5-, and 10-; 13- and 16-; 19- and 22-; 25- and 30-; and the 35-, 40-, and 45-min samples from both portal and jugular blood. Butyrate

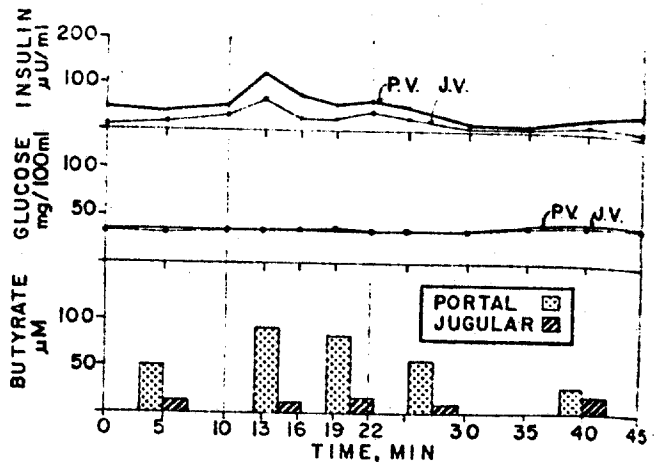

FIG. 3. Concentrations of blood components of *sheep 75* before, during, and after continuous infusion of 0.2 mmoles butyrate/min into the clamped anterior mesenteric artery. PV and JV refer to sampling from portal and jugular vein, respectively. The period between 10 and 22 min, as indicated by vertical lines, represents two separate periods of infusion.

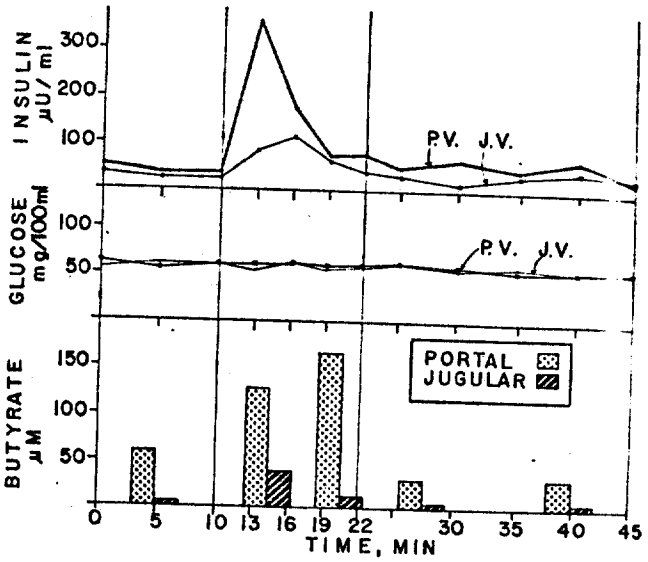

FIG. 4. Concentrations of blood components of *sheep 75* before, during, and after continuous infusion of 0.4 mmoles butyrate/min into the clamped anterior mesenteric artery. PV and JV refer to sampling from portal and jugular vein, respectively. The period between 10 and 22 min, as indicated by vertical lines, represents the period of infusion.

infusion at a rate of 0.2 mmoles/min caused no peripheral (jugular blood) elevation in butyrate, although portal vein concentrations were raised from an initial value of 50–90 μM. Portal and jugular vein concentrations of glucose remained constant throughout the experiment. Insulin in portal plasma increased from an initial concentration of 52 μU/ml to 120 μU/ml within 3 min. The jugular insulin curve followed a similar though less marked pattern. Figure 4 shows an experiment conducted with *sheep 75* 11 days postoperatively. It was performed exactly as the previous experiment except that the animal received 0.4 mmoles butyrate/min.

Blood glucose concentration remained constant whereas butyrate increased two- to threefold in portal blood. Butyrate in the pooled jugular plasma sample of 13 and 16 min was raised appreciably, but by 19 and 22 min only a slight increase was present. Insulin concentrations in portal plasma increased from 35 μU/ml to 360 μU/ml 3 min after initiation of the infusion. Jugular plasma insulin followed a similar though less striking increase.

The changes of mesenteric artery blood glucose, ketones, plasma insulin, and plasma butyrate concentrations after a single intravenous injection of 2.5 mmoles butyrate/kg into the jugular vein of *sheep 75* are shown in Fig. 5. Butyrate was injected over a period of 30 sec. Preinjection plasma butyrate concentration was 12 μM; at 5 min the level was 5.74 mM, but by 40 min the concentration was back to 63 μM. Insulin secretion was prompt and serum concentrations reached a peak of 1,290 μU/ml by 20 min. Concentrations of both total ketone bodies and β-hydroxybutyrate doubled, reaching a peak within 20–30 min. Control glucose concentration was 64 mg/100 ml 30 min after injection a concentration of 100 mg/100 ml was attained and by 120 min a reduction to 28 mg/100 ml occurred, presumably associated with the marked insulin secretion. Thirty-seven days after installation of the catheters *sheep 75* died, apparently from internal hemorrhage. At death, both catheters were open to infusion but samples could be collected from only the portal catheter. Autopsy revealed that the catheters and clamp had remained properly positioned.

On 11 November 1965, *sheep o*, a 60-kg wether, was prepared with two portal catheters. One arterial catheter was also placed about 4 cm into the dorsal aorta by way of the anterior mesenteric artery. Experiments were conducted on this and another animal (*sheep 145*) to compare insulin release following infusions into the portal vs. the jugular vein. The rationale was that, because butyrate is readily metabolized by the liver, infusion into the portal vein should result in only small elevations of butyrate in arterial blood reaching the pancreas (Figs. 3, 4), but would give a maximum potential for liver metabolites of butyrate. Conversely, a jugular infusion should give a maximum peripheral elevation of butyrate and a diminished opportunity for liver metabolites. Thus, if butyrate stimulated the pancreas directly, the jugular route of administration would be more effective, but if a liver metabolite was involved, the portal vein infusion would give a greater insulin secretion.

Figure 6 shows the result of infusing 0.5 mmoles butyrate/min into the portal vein of *sheep o* followed by infusion into the jugular vein at the same rate. Infusion via the portal vein increased arterial plasma butyrate concentration from 10 μM to about 60 μM; insulin concentrations in the portal vein increased slightly. Infusion into the jugular vein gave a butyrate elevation in arterial plasma from an initial concentration of 20 μM to about 70 μM, which was sufficient to increase portal plasma insulin concentration from 30 to over 400 μU/ml. Blood glucose in portal blood remained constant throughout.

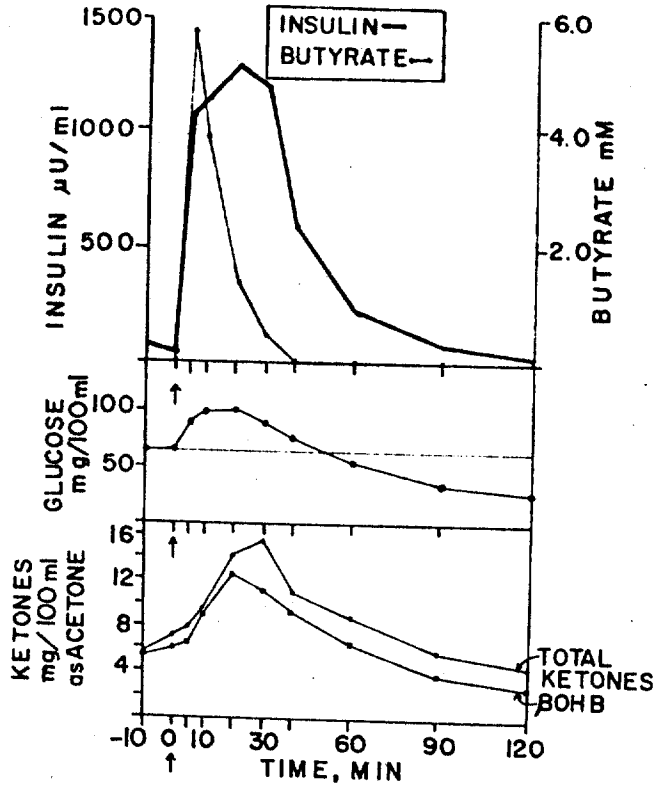

FIG. 5. Concentrations of anterior mesenteric artery blood components before and after single injection (arrows) of 2.5 mmoles butyrate/kg into jugular vein of *sheep 75*. βOHB refers to β-hydroxybutyrate.

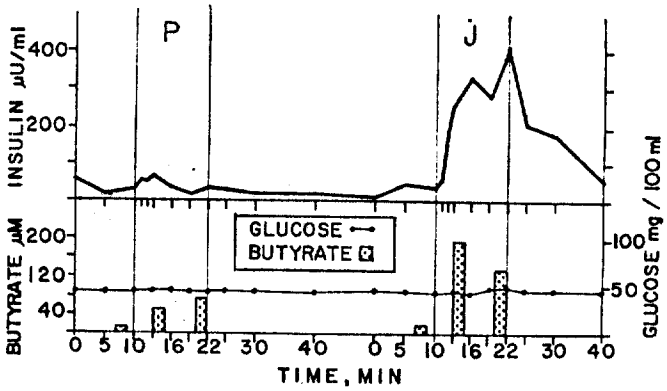

FIG. 6. Concentrations of portal vein blood glucose and insulin and arterial (aorta) butyrate of *sheep o* before, during, and after continuous infusion of 0.5 mmoles butyrate/min. P and J refer to portal and jugular veins, respectively, and indicate the routes of infusion. The periods between 10 and 22 min, as indicated by vertical lines, represent two separate periods of infusion.

On 24 November 1965, two portal catheters, one for infusion, the other for sampling, were installed in an 11-month-old ewe weighing 51 kg (*sheep 145*). The data of Fig. 7 demonstrate results of various rates of butyrate infusion into either the peripheral circulation (jugular) or to the portal vein. The data showed considerable

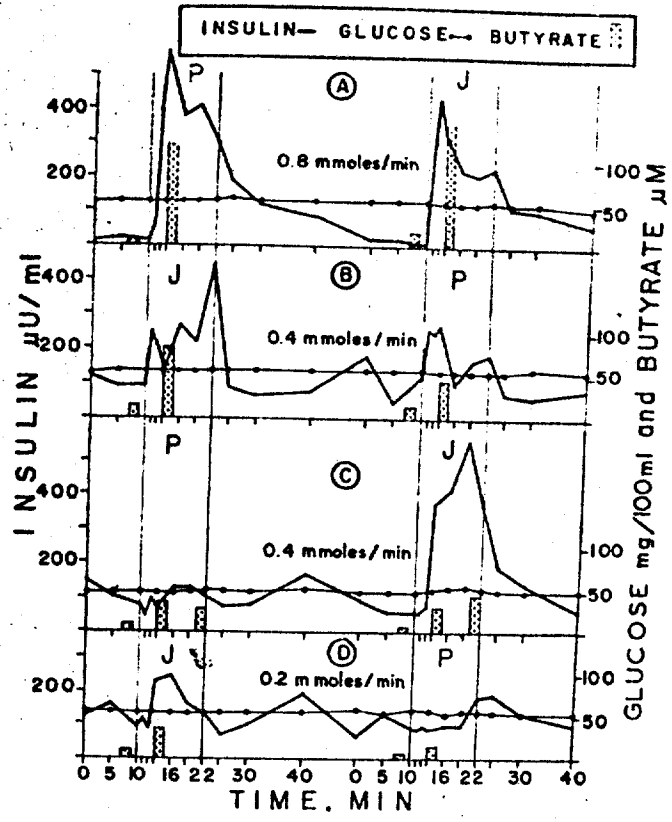

FIG. 7. Concentrations of portal vein blood glucose and insulin and jugular vein butyrate in four separate experiments (*A*, *B*, *C*, *D*) with *sheep 145* before, during, and after various rates of butyrate infusion. P and J refer to portal and jugular veins, respectively, and indicate the routes of infusion. The periods between 10 and 22 min, as indicated by vertical lines, represent two separate periods of infusion.

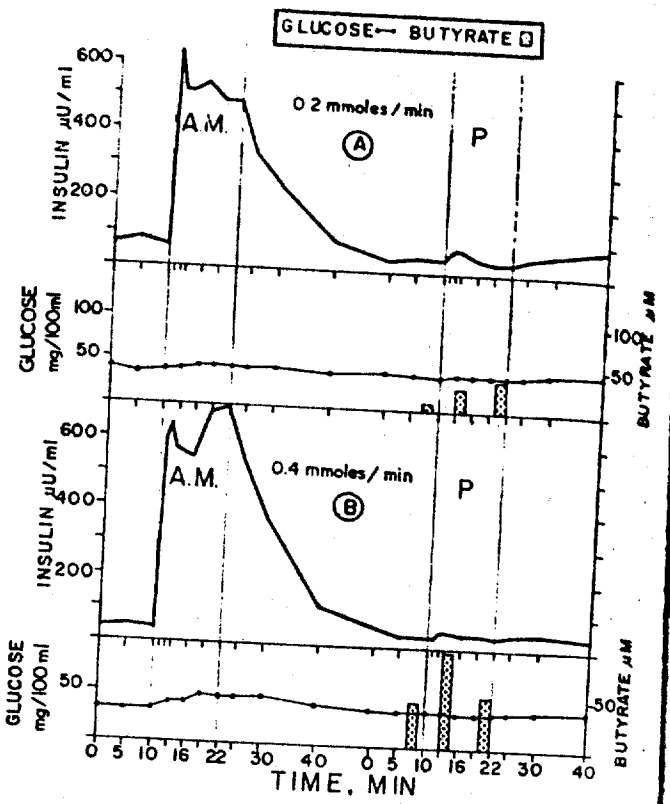

FIG. 8. Concentrations of blood components in two separate experiments with *sheep 968* before, during, and after continuous infusion of 0.2 mmoles butyrate/min (*exp. A*) and 0.4 mmoles butyrate/min (*exp. B*). A.M. and P refer to anterior mesenteric artery and portal vein, respectively, and indicate the routes of infusion. The periods between 10 and 22 min, as indicated by vertical lines, represent two separate periods of infusion. Butyrate was measured in arterial blood plasma, glucose and insulin in portal vein blood.

variation and the results are not easily interpreted because portal vein infusion in this animal generally resulted in peripheral elevations of butyrate even at low rates of introduction of exogenous butyrate. Higher concentrations of butyrate in peripheral blood were obtained by jugular infusion, however, and in general one must conclude that jugular infusion in this animal caused a greater insulin release than did portal infusion, thereby confirming the findings obtained with *sheep 0*.

The data obtained from experiments thus far were highly suggestive, but they did not provide adequate proof that butyrate could have a direct effect on the pancreas. Therefore, a wether weighing 58 kg (*sheep 968*) was prepared surgically on 31 December 1965 with two portal catheters; an anterior mesenteric artery catheter was also installed and the artery was fitted with a clamp at the bifurcation (Fig. 2). Figure 8*A* shows that administration of 0.2 mmoles butyrate/min directly into the blood supplying the pancreas increased portal insulin from an initial concentration of 62 µU/ml to 630 µU/ml within 1 min following beginning of infusion. Infusion into the portal vein caused a small peripheral elevation of butyrate as measured in anterior mesenteric artery blood, and insulin secretion increased slightly.

Figure 8*B* presents the results of an experiment similar to that of Fig. 8*A*, except that butyrate was infused at 0.4 mmoles/min. Glucose concentrations increased slightly, although a decrease might have been expected from the marked insulin release.

Figure 9 presents concentrations of glucose, insulin, and ketones measured in arterial blood in an experiment where 0.8 mmoles butyrate/min was infused into the portal vein of *sheep 968*. Acetoacetate was calculated as the difference between total ketones and β-hydroxybutyrate plus acetone. Total ketone bodies increased from about 2.5 mg/100 ml to nearly 4 mg/100 ml; β-hydroxybutyrate accounted for most of the increase. Ketone body increase was observed previously (5) with single injections of butyrate (2.5 mmoles/kg). Thus, one could not unequivocally rule out the possibility that this compound was of some importance in the insulin response to butyrate.

To investigate the possibility that increased ketone body concentrations were concerned with the butyrate-induced insulin release, β-hydroxybutyrate at rates of 0.4 and 0.8 mmoles/min were infused into the portal vein of *sheep 145* (Fig. 10*A*). Portal vein glucose and

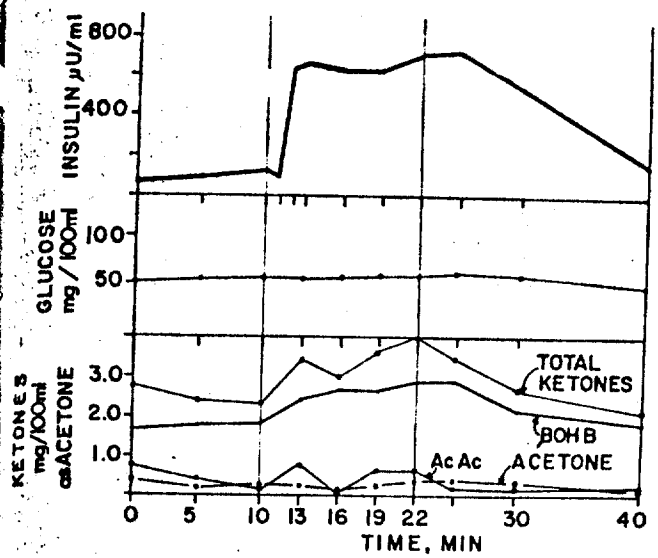

FIG. 9. Concentrations of anterior mesenteric artery blood components of *sheep 968* before, during, and after continuous infusion of 0.8 mmoles butyrate/min into the portal vein. The period between 10 and 22 min, as indicated by vertical lines, represents the period of infusion. βOHB and AcAc refer to β-hydroxybutyrate and acetoacetate, respectively.

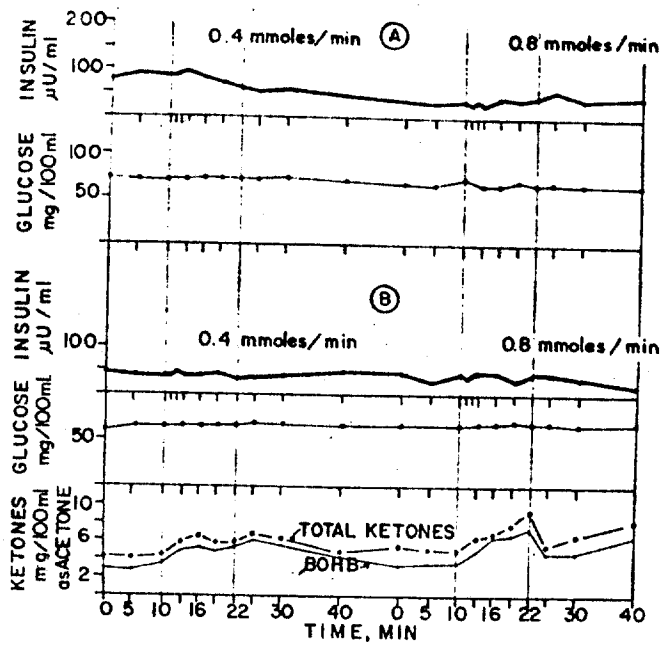

FIG. 10. *A:* concentrations of portal vein blood glucose and insulin before, during, and after continuous infusion of 0.4 and 0.8 mmoles βOHB/min into portal vein of *sheep 145*. *B:* concentrations of jugular vein blood glucose, insulin, total ketones, and βOHB before, during, and after continuous infusion of 0.4 and 0.8 mmoles βOHB/min into the opposite jugular vein of *sheep 3*. In each of the two experiments (*A*, *B*), the periods between 10 and 22 min, as indicated by vertical lines, represent two separate periods of infusion. βOHB refers to β-hydroxybutyrate.

insulin remained unchanged. A similar experiment with *sheep 3* (Fig. 10*B*) confirmed these results.

Two experiments were performed to verify that the surgical preparation allowed local enrichment of the blood supply to the pancreas and to compare release of insulin from butyrate with that resulting from glucose. These were similar and involved infusion of 5% glucose at a rate of 2.0 ml/min into the clamped anterior mesenteric artery. The results are shown for *sheep 75* and *968* in Fig. 11, *A* and *B*, respectively. The infusion failed to produce a definite increase in portal vein glucose and caused no change in jugular vein concentrations, but insulin release was unequivocal in both experiments. Insulin concentrations were elevated by 2 min in *sheep 968*, but not until 9 min after the start of infusion in *sheep 75*.

Physical obstruction of the artery by clamping caused no visible reaction in animals until 7–10 days after surgery, when they appeared uncomfortable. Since it seemed possible that clamping the artery could produce anomalous results, control experiments were performed with *sheep 75* and *968*. Saline infusions into the clamped anterior mesenteric artery of *sheep 75* indicated that the clamp itself did not affect results obtained. In both experiments with *sheep 968* (Fig. 8, *A* and *B*), the arterial clamp was inflated during infusion into the portal vein as well as into the artery. Consequently the only difference was the route of infusion, and it must be concluded that clamping the artery per se did not affect glucose or insulin concentrations.

Several experiments were conducted to determine if close to physiological entry rates of exogenous propionate had an effect on insulin concentrations. The infusion of 0.2 and 0.4 mmoles/min into the portal vein of *sheep 145* gave equivocal results (Fig. 12*A*), but a rate of 0.8 mmoles/minute (Fig. 12*C*) produced a striking increase in portal vein insulin concentrations. Infusion of 0.4 mmoles/min of propionate into the jugular vein of *sheep 3* (Fig. 12*B*) doubled jugular vein insulin concentrations and 0.8 mmoles/min produced a rapid and prolonged insulin elevation from 50 to 165 μU/ml. Glucose concentrations did not change in any of the above trials involving propionate.

The infusion of 2.0 mmoles acetate/min into the portal vein of *sheep 145* (part 1, Fig. 12*C*) did not increase insulin concentrations. The same rate of infusion into the jugular vein of *sheep 3* gave similar results. These two experiments and previous data (15) provide substantial evidence that acetate is not stimulatory to insulin secretion in sheep.

Experience with the four animals discussed (*sheep 75*, *145*, *0*, and *968*) represents varying degrees of success of operations performed on about 15 animals. Most failures resulted from death within 24 hr after surgery. Generally, trouble was experienced in placement of the clamp around the artery; damage to the common mesenteric vein was frequent in such instances. Two additional animals survived surgery, but the catheters became blocked so quickly that they could not be used in infusion experiments.

To test the validity of the in vivo data we attempted to employ the pancreatic incubation technique of Coore

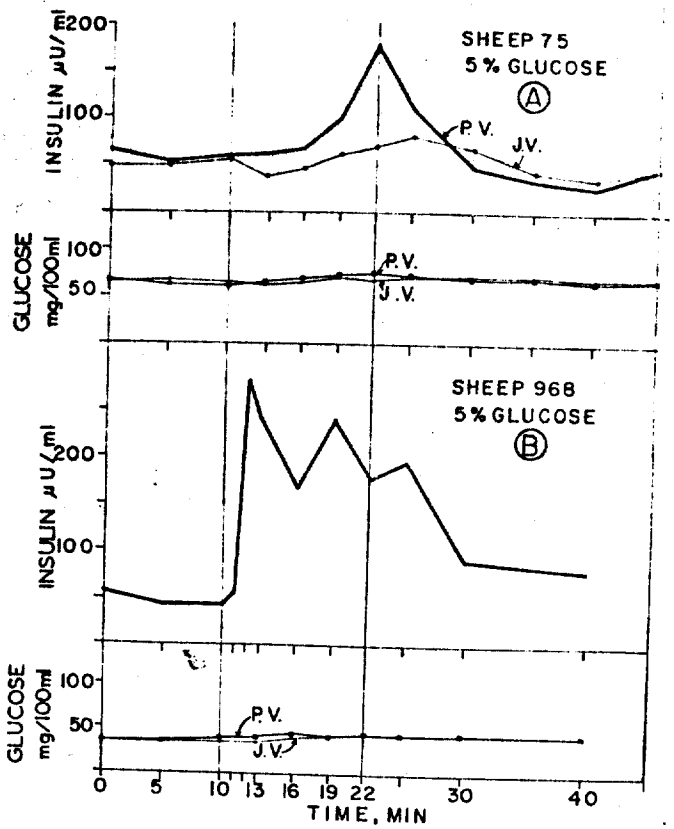

FIG. 11. *A:* concentrations of portal (PV) and jugular vein (JV) blood glucose and insulin before, during, and after continuous infusion of 5% glucose (2.0 ml/min) into the clamped anterior mesenteric artery of *sheep 75*. *B:* concentrations of portal vein (PV) blood glucose and insulin and jugular vein (JV) blood glucose before, during, and after continuous infusion of 5% glucose (2.0 ml/min) into the clamped anterior mesenteric artery of *sheep 968*. In each of the two experiments, the period between 10 and 22 min, as indicated by vertical lines, represents the periods of infusion.

and Randle (8). Direct application of this method to sheep pancreatic slices did not appear to be satisfactory. Under certain conditions insulin release to glucose was demonstrated but a consistent effect of butyrate was not observed. Additional work is required to establish proper conditions for the incubation system.

DISCUSSION

The fatty acid loads used in previous single injection experiments (15) were unphysiologically high and caution must be used in extrapolating these data to a physiological situation. Figure 5 demonstrates that injection of 2.5 mmoles butyrate/kg causes a plasma butyrate elevation of several hundredfold.

The surgical preparation employed in the present study which allowed infusions into the arterial supply of pancreas and into the portal vein was invaluable for assessing the influence of physiological rates of infusion and for determining whether butyrate had a direct effect on the pancreas. Initial acute experiments performed on anesthetized animals after preparatory surgery indicated that such experiments were not feasible if meaningful results were to be obtained. A major advantage of the chronic surgical preparation was that experiments could be performed on trained, unanesthetized animals.

Studies using animals with portal catheters indicated immediately the advantage of this sampling site over a peripheral one (e.g., jugular) with regard to the detection of insulin release. A given treatment could evoke a relatively large increase in portal vein insulin, but because of degradation in the liver and dilution in the general circulation, the peripheral insulin elevation would be small. For example, in Figs. 3 and 4 the jugular samples gave questionable increases after butyrate infusion, but assays on portal blood presented unequivocal results. Whether or not the increased plasma insulin concentrations after butyrate infusion can be accounted for by insulin secretion has been discussed in the preceding paper (15). In the present work (Figs. 6-8) up to about tenfold increases in portal vein insulin were recorded within 1-2 min after beginning the infusion. Considering the relatively long half-life of insulin in the circulation this result can be logically accounted for only by increased insulin secretion. In addition, if decreased insulin degradation accounted for the increase one would anticipate similar increases in portal and jugular vein insulin. In Fig. 4, 3 min after the butyrate infusion began, portal vein insulin increased sixfold compared to a threefold jugular vein increase. This indicates that portal vein insulin was being enriched by secretion from the pancreas.

The data gathered from experiments with *sheep 75* suggested strongly that butyrate could have a direct action on the pancreas. Butyrate infusions at these low rates did not evoke a hyperglycemic response. This possibility could therefore be discarded as an explanation for the insulin-releasing ability of butyrate in this animal. During the initial stages of experimentation with this sheep, the sensitivity of the pancreatic islets to butyrate was not known. To be a physiologically effective stimulant, butyrate would have to mediate the insulin response at the low concentrations that exist in the peripheral circulation (1) (Figs. 3, 4). Infusion of 0.2 and 0.4 mmoles butyrate/min into the anterior mesenteric artery (Figs. 3, 4) resulted in little or no elevation of butyrate in jugular plasma although portal and jugular vein insulin concentrations increased. However, infusion into the clamped anterior mesenteric artery presumably caused a butyrate elevation in pancreatic arterial blood in excess of that occurring normally. Subsequent experiments with *sheep 0* and *45* indicated that rates of infusion as low as 0.2 mmoles butyrate/min into the portal vein would promote insulin secretion. Although earlier reports (2) indicated little or no butyrate in arterial blood, the present work and that of others (1), using sufficiently sensitive methods of detection, indicate that concentrations of 10-50 μM are present in peripheral blood of ruminants. The

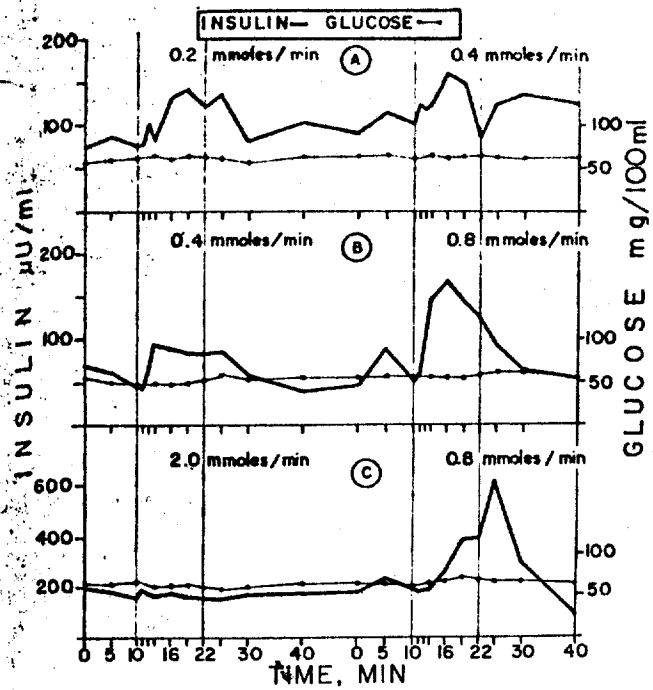

FIG. 12. Blood glucose and insulin before, during, and after continuous infusions in three sheep. *A:* portal blood samples; propionate infused at rates of 0.2 and 0.4 mmoles/min into portal vein of *sheep 145*. *B:* jugular vein samples; propionate infused at 0.4 and 0.8 mmoles/min into opposite jugular vein of *sheep 3*. *C:* portal vein samples; infusion of acetate (2.0 mmoles/min) and propionate (0.8 mmoles/min) into portal vein of *sheep 145*. In each of the three experiments (*A*, *B*, *C*) the period between 10 and 22 min, as indicated by vertical lines, represents two separate periods of infusion.

volatile fatty acid fraction of jugular vein plasma is about 70% as high as that of carotid artery plasma (13). Because this is a very low concentration it seems quite unlikely that butyrate per se is a quantitatively significant substrate for peripheral tissues. In addition, butyrate determination is tedious and time consuming and requires fairly large quantities of plasma; hence, investigators have largely ignored peripheral blood butyrate concentrations. Further, volatile fatty acid turnover is very rapid (half-life, 2–3 min) and consequently blood concentrations at any one instant could be quite meaningless. A more realistic approach to the problem is to measure entry rates from the rumen into the portal circulation over a longer period of time. Using radioisotope-infusion techniques Annison and Lindsay (3) and Bergman et al. (4) estimated butyrate entry rate from the rumen to be about 0.4 mmoles/min in sheep. Thus, the infusion rates in the present studies were low enough to allow the conclusion that butyrate could play a physiologically significant role in the regulation of insulin secretion in sheep. Using such low infusion rates, one must expect only moderate changes in serum insulin concentration and, ideally, samples of pancreatic venous blood should be used to detect increased secretion since considerable dilution occurs even in the portal vein. Presumably, several factors can alter insulin secretion by the pancreas (e.g., glucose, butyrate, propionate, epinephrine) and a change of butyrate concentration is only one of these; consequently it cannot explain all the changes of insulin secretion. Also, although the insulin assay is very sensitive and relatively precise, it is impossible to be sure that small differences of insulin concentration are not due to technical errors.

The experiments involving alternate infusion of butyrate, first into the jugular and then into the portal vein, and the experiments with *sheep 968* provide substantial evidence that butyrate has a direct effect on the pancreas. Infusion of β-hydroxybutyrate did not release insulin, providing evidence that butyrate does not act indirectly through its conversion to this ketone body. The results of experiments with *sheep 968* are striking because of the large increase attained in portal vein insulin (about 10-fold) within 1 min after beginning the butyrate infusion. Such a rapid response would also indicate a direct effect of butyrate.

Considerable evidence indicates that glucose must be metabolized, presumably intracellularly, to evoke a response (6, 7, 11, 16). Butyrate entering the portal circulation is considered to be metabolized primarily by the liver (5); to our knowledge, metabolism by pancreatic islet tissue has not been investigated. Butyrate concentrations as low as 30 μM (about 0.3 mg/100 ml) in jugular vein blood are associated with substantial insulin release (Fig. 7). Blood glucose concentration is about 200 times that of butyrate. Although the importance of increased intracellular concentrations of butyrate, or a butyrate metabolite formed in islet tissue, cannot be properly assessed at this time, the low effective concentration of butyrate does not indicate a major effect on intracellular metabolism in islet cells. The low effective butyrate concentration would, however, be consonant with a membrane effect of some type.

Recent work (9, 17) indicates that glucagon stimulates release of insulin. If this were the mechanism in the present study, butyrate would act initially on the glucagon-secreting cells, and insulin secretion would be secondary and dependent on glucagon release. Because of the close proximity of the islet cells which produce these two hormones, the response presumably could be fast (no longer than 1 min) and local concentrations of glucagon would be high. The temptation to implicate glucagon in this response is great, since it would explain the marked hyperglycemic action of single intravenous injections of butyrate. Further, with low rates of butyrate infusions, no hypoglycemia was produced even in the face of marked insulin secretion, and in some cases (e.g., Fig. 8 *A* and *B*) a slight increase in blood glucose resulted. This observation can also be interpreted as indirect evidence of concomitant glucagon release.

Although the data obtained using single injections of 2.5 mmoles propionate/kg in adult animals indicated that propionate was not stimulatory to insulin secretion (15), infusion of low levels was an effective stimulant to this process. Entry rate of propionate is over 1 mmole/min in fed sheep (3), and consequently this compound

could assume an important role in regulating insulin secretion. The experiments described here did not demonstrate that propionate had a direct effect on the pancreas. However, since the primary circulating metabolite would be glucose, which did not change during propionate infusion, it is most probable that this compound, like butyrate, has a direct effect on the pancreas.

These experiments have demonstrated that butyrate and propionate, two normal products of rumen fermentation, are stimulatory to insulin secretion in the sheep. The effect may be of physiological significance. Possibly considerable variation exists from species to species regarding the control of insulin secretion. Monogastrics such as the dog and human ingest and absorb large amounts of carbohydrate and concentration of blood glucose seems to be intimately associated with control of insulin secretion in such species. Ruminants such as the sheep absorb very little glucose from the digestive tract but produce large amounts of short-chain fatty acids. These compounds appear to be relatively more stimulatory than glucose to insulin secretion in the sheep.

The concept of an important role of propionate and butyrate in controlling insulin secretion in sheep automatically places importance on the degree of extraction of these two compounds from portal blood by the liver. The arterial levels of butyrate and propionate are a direct consequence of their concentrations in portal blood and the amounts removed by the liver. Removal of these compounds by the liver probably is not constant and depends on blood flow, the abundance of intracellular metabolites necessary for propionate and butyrate metabolism, and other factors. If one considers a hypothetical situation which might obtain if volatile fatty acids were being produced at a rapid rate by the rumen, propionate and butyrate would be taken up avidly by the liver. Gluconeogenesis would be accelerated until normal liver glycogen stores were replete. At this point, liver extraction of propionate and butyrate would decrease somewhat and arterial levels would increase, providing the stimulus for insulin (and possibly glucagon) release. The excess glucose synthesized by the liver during the period of increased insulin concentrations would be transferred into peripheral tissues under the influence of insulin. If both insulin and glucagon were released simultaneously, liver glycogenolysis would occur, accompanied by increased transfer of glucose into peripheral tissues; glucose turnover would be increased simultaneously.

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Insulin release by acetate, propionate, butyrate, and glucose in lambs and adult sheep[1]

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MANNS, J. G., AND J. M. BODA. *Insulin release by acetate, propionate, butyrate, and glucose in lambs and adult sheep.* Am. J. Physiol. 212(4): 747–755. 1967.—Experiments were designed to define the role of glucose in controlling insulin secretion in sheep and to determine if the major products of rumen fermentation, namely, acetic, propionic, and butyric acids have a function in such control. Intravenous injections of glucose (0.56, 1.11, and 1.67 mmoles/kg) into four adult sheep did not increase serum insulin concentrations statistically different from the saline control. Injections of butyrate and propionate produced similar hyperglycemias when administered intravenously (2.5 mmoles/kg). Butyrate, but not propionate, evoked a rapid, sustained elevation of serum insulin not explained by the associated hyperglycemia. Acetate at the same dose did not affect blood glucose or serum insulin. Intravenous butyrate injection (2.5 mmoles/kg) into seven lambs 2, 6, 12, and 24 weeks of age elevated serum insulin concentrations more than a similar or greater hyperglycemia induced by glucose (1.25 mmoles/kg). Propionate (2.5 mmoles/kg) evoked insulin release as great and probably greater than glucose, but considerably less than butyrate. As lambs grew older, there appeared to be a reduced sensitivity of the insulin secretory apparatus to stimulation by glucose.

volatile fatty acids; glucose tolerance in relation to age; diurnal variation of insulin and glucose; insulin immunoassay in sheep

FOLLOWING THE DEVELOPMENT of a specific and precise technique for measuring insulin in biological fluids (39), considerable effort has been directed toward elucidating those factors which control insulin secretion. A large body of evidence (for reviews see 12, 40) indicates that the concentration of glucose in the arterial blood reaching the pancreatic islets is associated with regulation of insulin secretion.

Certain other hexoses can also result in insulin release (6, 14). This ability of several sugars to stimulate insulin release and data indicating that mannoheptulose, a compound which inhibits glucose phosphorylation, at least in liver cells (9), and also blocks insulin release in response to glucose (8), suggests that an intracellular metabolite(s) is involved. The report (20) that ketone bodies can stimulate insulin release in the dog could also be ascribed to an intracellular effect. However, recent reports demonstrating that epinephrine inhibits (10) and glucagon (35) and corticotrophin (13) stimulate insulin release suggest a different level of control.

The sheep pancreas will secrete insulin in response to marked experimental hyperglycemia (6), but it is not known whether this is physiologically important or if other means of control are utilized. Schambye (36) studied blood glucose concentrations of sheep fed every 12 hr. Hourly blood sampling gave almost constant concentrations of glucose in portal vein and carotid artery blood from 0 until 12 hr after feeding. Annison, Hill, and Lewis (3) fed animals once daily and observed a tendency for blood glucose to increase only slightly beginning at 6–8 hr postfeeding. The change was from an initial concentration of about 45 mg/100 ml to 55 mg/100 ml and occurred over a period of 2–3 hr.

The sheep derives a large part of its supply of metabolic substrates from end products of rumen fermentation. The three major volatile fatty acids produced and absorbed into the portal circulation are acetic, propionic, and butyric (1). Significant changes in rumen and portal blood concentrations of these acids are associated with feeding, although peripheral concentrations are low and seem to be quite constant (3, 36). Experiments were designed to test the hypothesis that these volatile fatty acids, quantitatively very significant to the sheep, are more concerned with the control of insulin secretion than glucose, since there is considerable doubt as to whether the small fluctuations and typically low concentrations of blood glucose would be an adequate control mechanism for insulin secretion. An abstract of a portion of this work has been published (21).

MATERIALS AND METHODS

Four mature wethers 3–4 years of age were used to study the effect of three glucose loads on serum insulin concentrations. The animals were housed indoors and

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fed a maintenance ration of alfalfa pellets once daily between 8 and 9 AM. Animals were not fed the day before or the day of the experiment. Glucose was prepared as a 50% solution in 0.9% saline and given intravenously to each animal at doses of 0.56, 1.11, and 1.67 mmoles/kg (0.1, 0.2, and 0.3 g glucose/kg). To complete the 4 × 4 Latin-square design each animal received 0.9% saline in control experiments. Samples of jugular blood were taken by venipuncture at 0, 5, 10, 20, 40, 60, 90, 120, and 150 min after injection and were analyzed for whole blood glucose and serum insulin. Experiments were conducted 7 days apart and each animal was used on each of the 4 days of experimentation.

To obtain information on the magnitude of diurnal variation of bood glucose and serum insulin, a repetitive study was made involving four adult wethers 2–4 years of age. The sheep were housed indoors and fed a maintenance ration of alfalfa pellets containing 5% barley once daily at 8 AM. The animals finished 95% of their feed allotment within 1.5 hr. Each week for 4 consecutive weeks all four sheep were bled by jugular venipuncture at −½, 0, 1, 2, 3, 4, 6, 8, 10, 12, and 24 hr before and after feeding.

To study the effect of volatile fatty acids on serum insulin concentration, four ewes 2–5 years of age were used. They were housed indoors and were fed a maintenance ration of alfalfa pellets once daily between 8 and 9 AM. The day before and the day of the experiment feed was withheld. All experiments were made in the morning. Acetate, propionate, and butyrate solutions neutralized to pH 7.0 with sodium hydroxide at a final concentration of 4.0 M were each given as a single intravenous injection at a dose of 2.5 mmoles/kg. Blood samples were taken by jugular puncture at −30, −15, 0, 5, 10, 20, 30, 40, 60, and 90 min. Injection was at time 0 immediately after the last of the three control samples. One

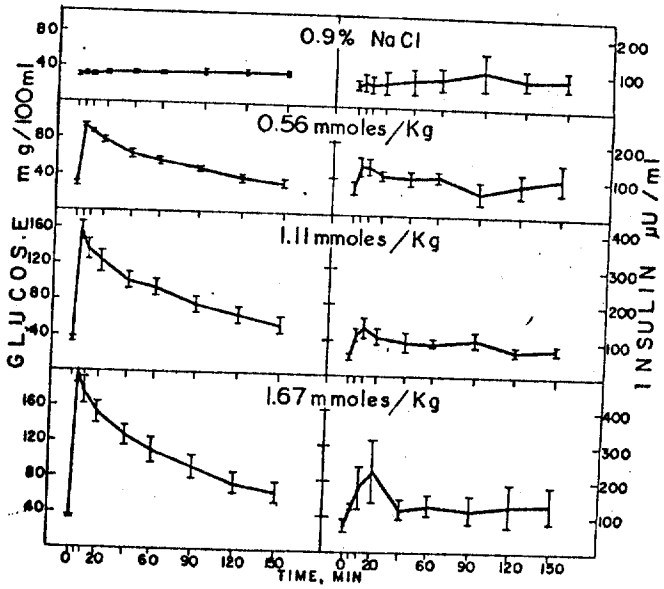

FIG. 1. Means and SE's of blood glucose and serum insulin concentrations before and after injection (time 0) of three dosages of glucose and saline into each of four adult sheep.

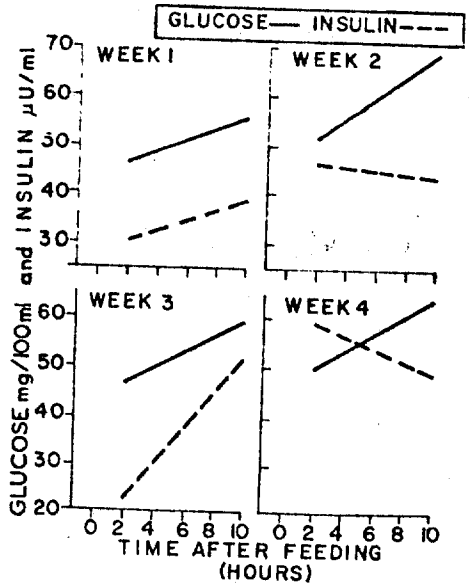

FIG. 2. Regressions of mean blood glucose and serum insulin concentrations with time after feeding in four adult sheep. Probabilities of the regression equations for the 4 weeks which were statistically significant or approached significance were as follows: *week 1*, glucose <0.10; *week 2*, glucose <0.20; *week 3*, glucose <0.05, insulin <0.025; *week 4*, glucose <0.025.

aliquot of each blood sample was heparinized for plasma free fatty acid analysis; a protein-free filtrate was prepared on a second aliquot and used for glucose and total ketone body analysis. Serum was separated from each sample and frozen for insulin assay. Two animals received 2.5 mmoles sodium chloride/kg in control experiments.

To study changes in pancreatic activity in response to propionate, butyrate, and glucose as a function of age, experiments were conducted with seven lambs: three sets of twins (*numbers 31, 32; 41, 42; 109, 110*) and one single (*number 145*). Injections were made at 12–16, 42–46, 84–88, and 168–172 days of age. For brevity these will be referred to as 2, 6, 12, and 24 weeks. Legends of the appropriate figures indicate the animals used in each treatment. Throughout all experiments using a single intravenous injection of butyrate, an animal would occasionally exhibit symptoms of severe respiratory stress. *Lamb 110* at 2 weeks and *lamb 41* at 24 weeks died from the treatment. Dosages for propionate, butyrate, and saline were 2.5 mmoles/kg; glucose was given at a dose of 1.25 mmoles/kg. These dosages of fatty acids had been used in related studies (18, 29, 30), and it seemed desirable to employ a dosage which had previously been investigated to facilitate interpretation of our data. All compounds were administered as a single intravenous injection via a jugular catheter. At 2 and 6 weeks of age, animals were fasted for 4 hr; at 12 and 24 weeks, a 12-hr fast was imposed. Lambs were weaned at 14 weeks.

Samples were taken from the jugular catheter at 0, 7.5, 15, 25, 35, 60, and 90 min after injection into the

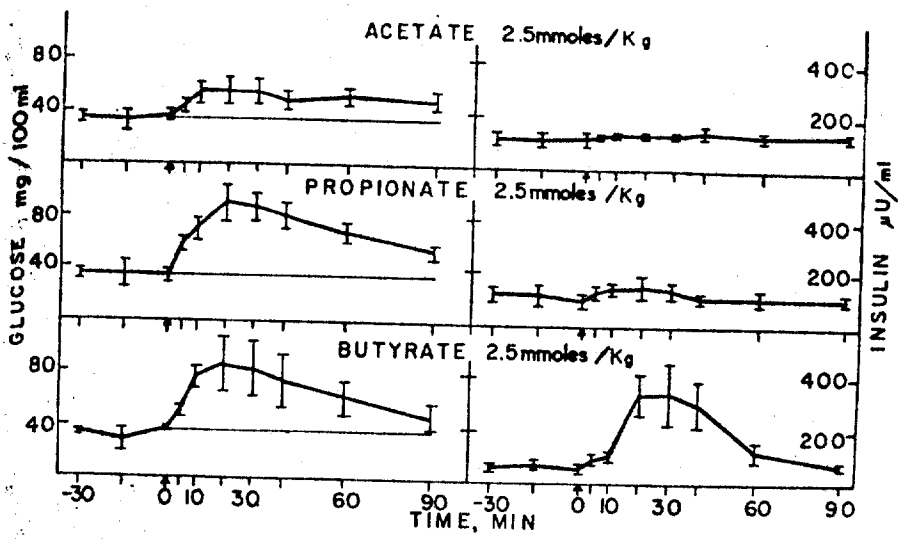

FIG. 3. Means and SE's of blood glucose and serum insulin concentrations before and after injection (arrows) of volatile fatty acids into four adult sheep.

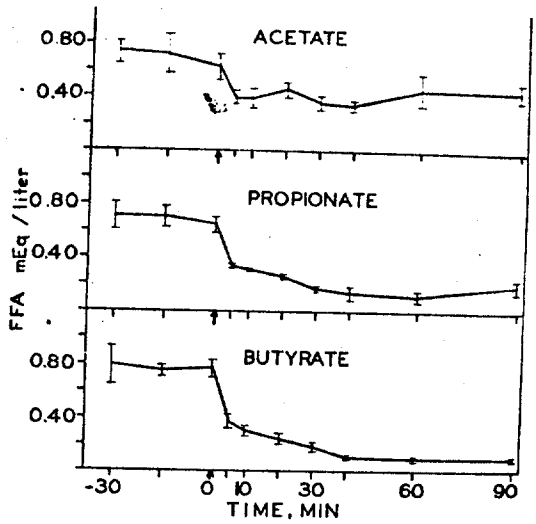

FIG. 4. Means and SE's of plasma free fatty acid (FFA) concentrations (mEq/liter) before and after injection (arrows) of equimolar amounts (2.5 mmoles/kg) of acetate, propionate, and butyrate.

2-week-old animals. An additional sample was collected at 45 min in subsequent age periods. Glucose was determined on a protein-free filtrate; insulin assays were performed on serum samples.

In all experiments where applicable, blood glucose was estimated on protein-free filtrates (37) of whole blood using a glucose oxidase reagent (Glucostat, Worthington Biochemical Corp., Freehold, N.J.). Plasma free fatty acids were determined by a modification (2) of a method described by Dole (11). Acetone and β-hydroxybutyrate were estimated by the method of Peden (27). Total ketone bodies were determined by a slight modification of Peden's method in that no acidification and heating step was used to remove acetone and acetoacetate.

Insulin was assayed by a modification of the double antibody immunoassay (26). Insulin antibodies to beef and pork insulin were developed in guinea pigs by the method of Robinson and Wright (33); the rabbit antiserum to guinea pig globulin was prepared using guinea pig γ-globulin (15). The principles involved in using the immunoassay have been reviewed (5, 35). Throughout the entire procedure, the tubes were agitated at about 4 C in a mechanical shaker. The assay was performed by adding the components in the following order:

a) Insulin standards (0.2 ml) and unknown serum or plasma. When less than 0.2 ml of unknown serum was used, the volume was adjusted to 0.2 ml with serum from an alloxanized sheep.

b) Four-tenths milliliter of an appropriate dilution of anti-insulin antibody prepared in 0.1 N barbital buffer, pH 8.6, containing 1.25 mg/ml bovine serum albumin (34).

c) Five microunits high specific activity porcine insulin ^{131}I (Abbott) in 0.4 ml barbital buffer 6–24 hr after the antibody and standard or unknowns.

d) One-tenth milliliter rabbit antiserum to guinea pig γ-globulin 12–24 hr after addition of insulin ^{131}I.

e) One-tenth milliliter 1:100 normal guinea pig serum 3–4 hr after the addition of the precipitating antibody.

Five to 24 hours after the addition of *e*, the tubes were centrifuged for 20 min at 13,000 *g* at 4C. The supernatant fluids were decanted and 1.1 ml barbital buffer were placed in each tube. After a further centrifugation for 10 min the supernatant fluids were again discarded, and the precipitates were counted in a well-type crystal-scintillation counter. Standards of crystalline sheep insulin were prepared once a week, using serum from an alloxanized sheep as a diluent (22). Standards of 0, 10, 20, 40, 60, and 100 μU/0.2 ml were analyzed in triplicate, unknowns in duplicate, for each assay.

RESULTS

The effectiveness of exogenous glucose and saline in altering serum insulin concentrations is shown in Fig. 1. Glucose concentrations were appreciably elevated by all

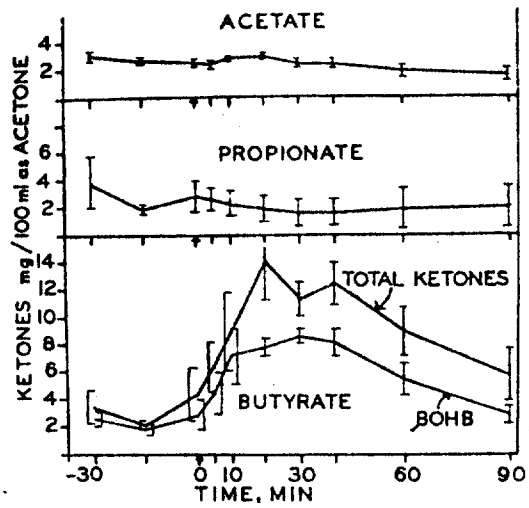

FIG. 5. Means and SE's of total blood ketone body concentrations (mg/100 ml as acetone) before and after injection of equimolar amounts (2.5 mmoles/kg) of acetate, propionate, and butyrate. In addition to total ketones, β-hydroxybutyrate (βOHB) was measured after butyrate injection.

three doses of glucose. Although a definite trend for insulin values to be increased after glucose injection was present, particularly at a dose of 1.67 mmoles glucose/kg, the increase of serum insulin concentration was of small magnitude and the variation in response between animals was so great that analysis of variance indicated no differences from saline control values at the 5% level of probability. Increases of serum insulin 10 and 20 min after injection of 1.67 mmoles glucose/kg did approach the 10% level of significance and, most likely, if more animals had been used the 5% level of significance would have been reached. These data confirm the low glucose tolerance of the adult sheep (17, 24) and point out its association with a relative insensitivity of the pancreatic islets to elevations of blood glucose.

In the repetitive study of diurnal variation, there were no changes of either serum insulin or blood glucose concentrations immediately before or during the first 3 hr after feeding. Therefore, presentation of the data was simplified by omitting the values obtained before feeding (−½ and 0 hr) and by averaging the 1-, 2-, and 3-hr postfeeding values, the means of which are referred to as the 2-hr samples. Variance analysis of the data indicated significant differences between the 4 weeks of sample collection, but not between animals at any 1 week in the changes of insulin and glucose with time after feeding. Regression lines of mean glucose and insulin concentrations between 2 and 10 hr after feeding were calculated for the four sheep at each period and are presented in Fig. 2. Statistical analysis of the regression equations indicated that blood glucose concentrations increased substantially between 2 and 10 hr after feeding. However, a concomitant elevation of serum insulin occurred in only one of the four periods (*week 3*). Twenty-four hours after feeding, both glucose and insulin returned to approximately prefeeding values.

The relative effects of intravenous injections of acetate, propionate, and butyrate on blood glucose and serum insulin concentrations are shown in Fig. 3. Free fatty acids (FFA) and ketone bodies were also measured and are presented in Figs. 4 and 5, respectively.

The hyperglycemias produced by propionate and butyrate were similar, but propionate caused only a slight increase in serum insulin whereas butyrate injection promoted a pronounced and sustained increase of serum insulin concentrations much in excess of that expected from the degree of hyperglycemia. The data of Fig. 1 provide additional justification for disregarding blood glucose elevation as being the cause of the insulin response to butyrate. Acetate injection produced an increase in blood glucose of about 20 mg/100 ml. Such an elevation, although minor for some species, is appreciable for adult sheep. However, insulin values did not increase either as a result of this hyperglycemia or of acetate per se. The injection of sodium chloride into two control sheep in amounts comparable to the volatile fatty acids administered did not affect blood glucose or serum insulin concentrations.

Plasma free fatty acids (Fig. 4) were reduced within 5 min after acetate, propionate, and butyrate treatments. This reduction was particularly striking in the cases of propionate and butyrate and this effect was still present 90 min after injection. Total ketone bodies were measured on whole-blood filtrates and are shown in Fig. 5. Acetate had no effect on total ketones, but propionate caused a reduction by about 20 min after injection which lasted until the 90-min sample. Butyrate produced an immediate elevation in ketone bodies. Figure 5 also shows the levels of β-hydroxybutyrate following butyrate injection. A significant portion of the increase in total ketones can be accounted for by the production of this ketone body. Injections of 2.5 mmoles sodium chloride/kg into two animals did not affect FFA or total ketone bodies.

Changes of glucose and serum insulin concentrations in response to propionate, butyrate, and glucose injections into lambs at various ages are presented in Figs. 6–11. The blood glucose elevations resulting from injection of propionate shown in Fig. 6 demonstrate an initial slight hyperglycemia at all ages; this is most evident at 24 weeks. Blood glucose concentrations returned to preinjection values sooner at 2 weeks than at other ages and from 25 to 90 min after injection a pronounced hypoglycemia was produced in 2-week-old lambs. However, Fig. 7 shows that insulin concentrations at 2 weeks were only slightly greater than at 6 and 12 weeks and almost the same as at 24 weeks. It should be pointed out that *lamb 42* at 2, 6, and 12 weeks responded to propionate with an increase in serum insulin of only about half of that observed in the other two lambs receiving propionate. At 24 weeks, however, *lamb 42* exhibited insulin concentrations about two times that of the other two lambs, thus giving what may be only an apparent high insulin elevation at 24 weeks. Figure 8 presents changes of blood glucose following butyrate

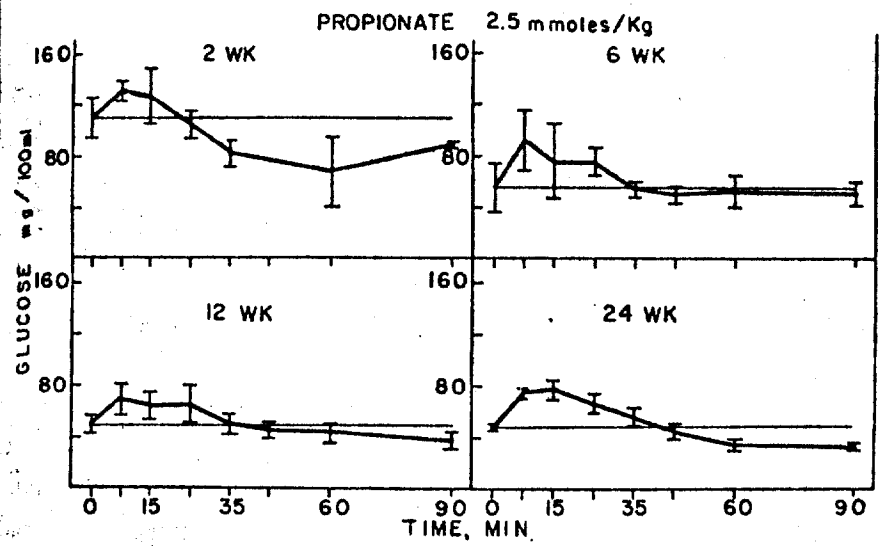

FIG. 6. Means and SE's of blood glucose concentrations before and after injection (time 0) of propionate into three lambs as affected by weeks of age. Lambs used: 2 weeks—*109, 42, 110*; 6, 12 and 24 weeks—*109, 42, 32*.

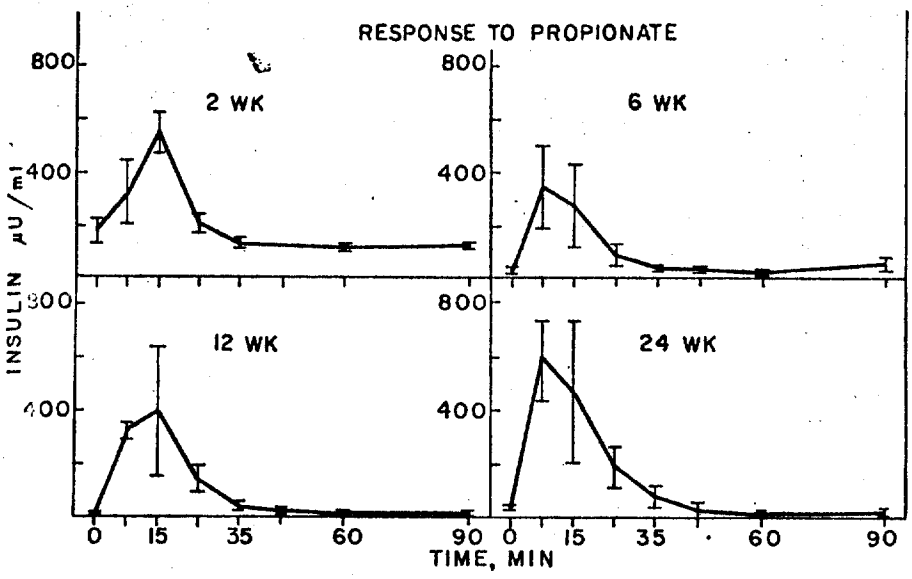

FIG. 7. Means and SE's of serum insulin concentrations before and after injection (time 0) of propionate into three lambs as affected by weeks of age.

injection. The curves are similar to the propionate curves (Fig. 6), but at each period there was a very definite and prolonged hypoglycemia, presumably associated with the marked increases of serum insulin at all ages as shown in Fig. 9. Figure 10 shows the hyperglycemia produced by 1.25 mmoles glucose/kg. Increases in glucose concentration at the 7.5-min sampling were 1.6-, 2.0-, 2.5-, and 2.5-fold the preinjection value at 2, 6, 12, and 24 weeks of age, respectively. Glucose concentrations had declined to preinjection values 25–35 min after injection at 2 weeks but at 6, 12, and 24 weeks 60–90 min were required. By comparison an injection of 1.11 mmoles glucose/kg into adult animals (Fig. 1) gave about 3.8-fold elevation at 7.5 min and had not declined to preinjection levels by 150 min. The insulin response to glucose (Fig. 11) appeared to be greater at 2 weeks than at other ages.

DISCUSSION

The paucity of information concerning the control of insulin secretion in sheep was alluded to in the introduction. Glucose is an important metabolic substrate for sheep as well as other animals, but products of rumen fermentation assume major significance in this and other ruminant species. The present experiments were designed to define the role of glucose in the control of insulin secretion in sheep and to determine if the major products of rumen fermentation, namely, acetic, propionic, and butyric acids had a function in such control.

Boda (6) reported that marked experimental hyperglycemia evoked prolonged elevations of serum insulin in sheep. In the present work, the hyperglycemias induced were moderate, although even the lowest dosage of glucose (0.56 mmoles glucose/kg) gave elevations in

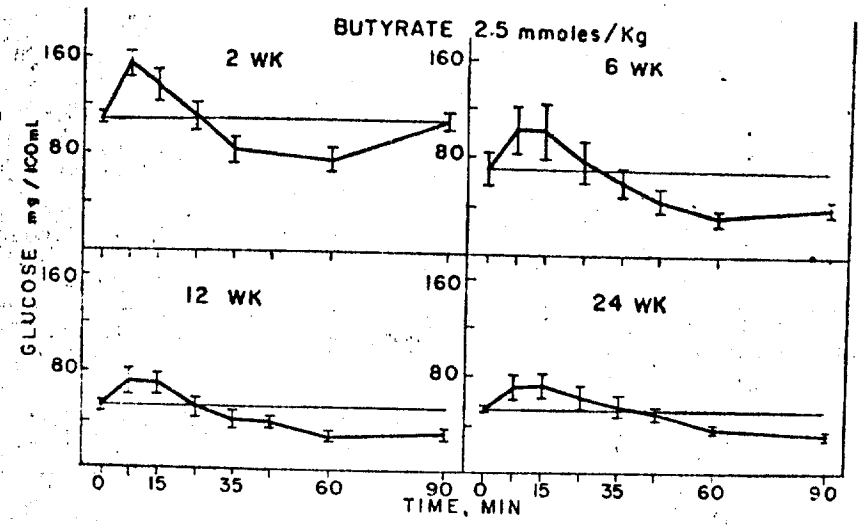

FIG. 8. Means and SE's of blood glucose concentrations before and after injection (time 0) of butyrate into four lambs as affected by weeks of age. Lambs used: 2, 6, and 12 weeks—*109, 145, 31, 41*; 24 weeks—*109, 145, 31, 32*.

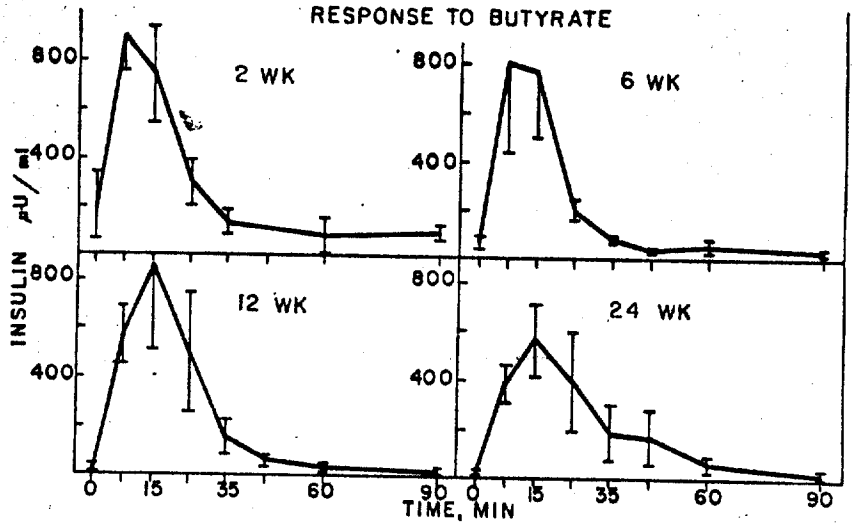

FIG. 9. Means and SE's of serum insulin concentrations before and after injection (time 0) of butyrate into four lambs as affected by weeks of age.

the maximum normal range for adult sheep. Despite this, statistically significant increases in serum insulin were not obtained at any of the dosages employed although a definite trend in this direction was observed. A larger number of experimental animals most likely would have demonstrated a significant difference at least at the 1.67-mmoles glucose/kg dose.

The diurnal variation experiment indicated that on a once-a-day feeding regimen blood glucose increased over a 2- to 10-hr postfeeding period. But, in only one of four identical studies was there a significant concomitant insulin elevation. Continuous access to feed probably would decrease the magnitude of postprandial fluctuations of blood glucose and consequently there would be even less likelihood of finding a correlation between blood glucose and serum insulin under nonexperimental conditions. More meaningful results would have been obtained had portal samples been used for insulin assay. Degradation of insulin takes place primarily in the liver (25); consequently, peripheral levels reflect the amount escaping liver degradation. Thus, increased insulin release could be countered by increased liver degradation, with peripheral levels remaining relatively stable. Additional experiments are required to establish firmly the role of fluctuations of blood glucose, but data presented here suggest this compound has a minor role under normal conditions.

It is difficult to obtain conclusive experimental evidence concerning the influence of a constant concentration of blood glucose on insulin release. Does the pancreas secrete insulin at a certain basal rate in response to a particular constant concentration of blood glucose, or is a small but definite change in glucose concentration necessary? Conversely, does the pancreas have a built-in autonomy which operates uninfluenced by glucose or other compounds?

The hyperglycemic action of propionate and butyrate in the ruminant has been well documented (30, 32). Propionate is a glucogenic substrate and propionate carbon is readily incorporated into blood glucose in vivo (4) and into glucose by liver slices in vitro (19). Both of these reports also showed that butyrate carbon was not incorporated into glucose in sufficient amounts to account for increased blood glucose in vivo. Phillips et al. (29)

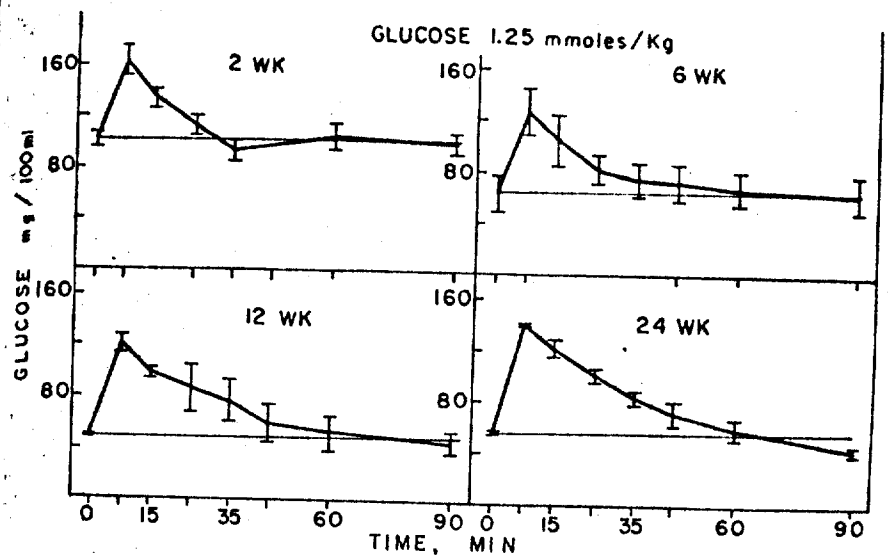

FIG. 10. Means and SE's of blood glucose concentrations before and after injection (time 0) of glucose into three lambs as affected by weeks of age Lambs used: 2 weeks—*145, 32, 109*; 6, 12, and 24 weeks—*145, 32, 42*.

proposed an indirect mechanism for the glucogenicity of butyrate involving liver phosphorylase activation, namely, that blood glucose arises from liver glycogenolysis. In present experiments, single injections of acetate, propionate, and butyrate into adult animals produced the anticipated hyperglycemia with propionate and butyrate. However, the serum insulin concentrations produced by butyrate were much greater than those with propionate, despite the very similar degrees of hyperglycemia. Increased serum insulin concentrations associated with propionate and butyrate injection are most logically accounted for by increased secretion of insulin by the pancreas. However, appreciable increases could be observed if the destruction of insulin were decreased by these compounds. In Figs. 7 and 9, serum insulin concentrations were increased 10- to 15-fold 7.5 min after injection in several experimental periods. The half-life for insulin in the circulation is in the order of 20–30 min in man, rabbit, and rat (25, 40). Sheep insulin has a similar half-life in the circulation (Boda, unpublished). Therefore it does not appear that the marked increases of serum insulin concentration observed could be explained by decreased destruction of insulin since the long half-life of insulin in the circulation in relation to the sampling time (7.5 min) would preclude this possibility.

In the course of maturation, ruminants such as sheep undergo a change from a monogastric-like animal in the immediate postnatal period to one which derives its primary metabolic substrates from products of rumen fermentation. Current evidence indicates that control of insulin secretion in monogastrics is associated with fluctuations of blood glucose concentrations. Following the demonstrations that the adult sheep pancreatic islets were relatively insensitive to the concentration of circulating glucose, it was of interest to determine if the young differed in this respect. Glucose, propionate, and butyrate produced marked insulin secretion in 2-week-old lambs. The response to glucose appeared to diminish somewhat by 6 weeks and presumably eventually would have reached the low level observed for mature adults. In the period from birth to about 3 weeks, lambs depend almost exclusively on readily absorbable milk constituents for their metabolic needs. By about 6 weeks, the rumen becomes of functional significance (38). The blood glucose concentration drops from over 100 to 50–60 mg/100 ml during this phase. Also, coinciding with this period of change from a monogastric-like to a ruminant animal, the tolerance to a glucose load decreases (17, 24). Chesrow and Bleyer (7) described decreased glucose tolerance in elderly humans and Zhukov (41) reported experiments with humans and rats which indicated that the pancreatic islets became less responsive in middle and old age. Therefore the current observations may indicate decreased sensitivity of the insulin secretory apparatus in adult sheep compared with lambs and this could be a partial explanation of the decreased glucose tolerance accompanying maturity.

These experiments with lambs of varying age periods confirmed the data from adult sheep, namely, that butyrate-induced hyperglycemia was not the explanation for the insulin release associated with injection of this compound. The data demonstrated a marked insulin release with only minor hyperglycemia. A reduction of blood glucose below the preinjection concentration subsequent to the initial hyperglycemia was particularly evident at 2 and 6 weeks of age. This fall in blood glucose to values lower than in the control period was not observed with mature animals (Fig. 3), and this may indicate decreased tissue sensitivity to insulin by adults.

Butyrate injection increased ketone body concentrations appreciably in every instance. The ketogenic effect of butyrate has been described by several workers (16, 28, 31). On the basis of the report that ketone bodies stimulated insulin secretion in the dog (20), ketone body-mediated butyrate stimulation was a distinct possi-

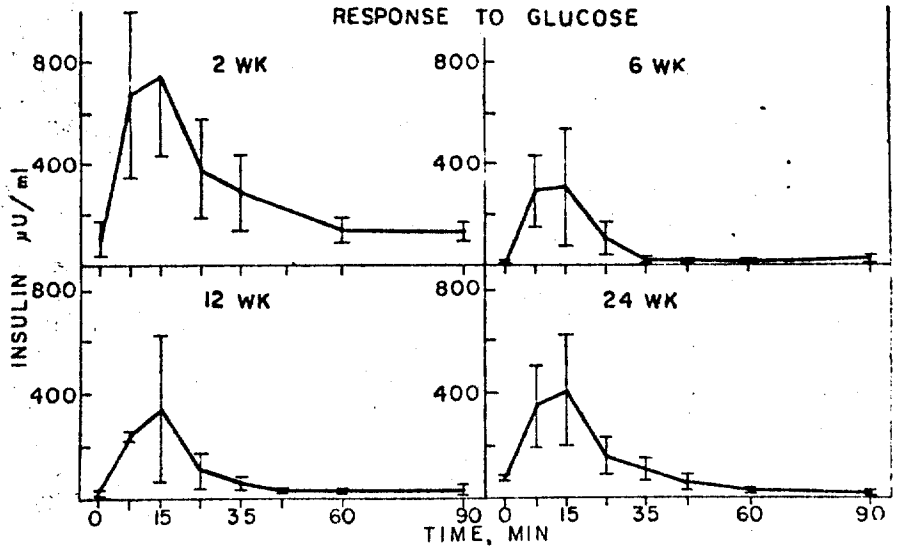

FIG. 11. Means and SE's of serum insulin concentrations after injection (time 0) of glucose into three lambs as affected by weeks of age.

bility. However, further data to be discussed in a subsequent report refuted such a mechanism (23).

Experiments with propionate in adult animals did not indicate an insulin stimulatory effect of propionate per se, that is, one which could not be accounted for by the induced hyperglycemia. Although the data for lambs could be interpreted in a similar way, a somewhat greater insulin secretion could be detected in the propionate experiments than could be accounted for by the resultant hyperglycemia.

The elevations in concentrations of acetate, propionate, and butyrate in plasma produced by the single intravenous injections undoubtedly exceeded physiological concentrations manyfold. For example, in an experiment similar to those reported here (23) a single intravenous injection of 2.5 mmoles butyrate/kg resulted in an increase in plasma butyrate concentration from 12 μM to 5.74 mM. It is obvious therefore that the present results indicating release of insulin with butyrate and propionate must be interpreted with caution. A subsequent report (23) describes experiments using more physiological concentrations of volatile fatty acids.

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Propionic acid as a precursor in the biosynthesis of animal fatty acids*

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SUMMARY

The carboxyl carbon of propionate was found to be a poor precursor for the synthesis of fatty acids in the rat. The data indicate that propionate as a three-carbon unit is not incorporated into long-chain fatty acids by the intact rat to any appreciable extent. The results also suggest that the conversion of propionate to long-chain fatty acids in surviving adipose tissue occurs primarily by a mechanism involving the decarboxylation of the propionate. These data are consistent with the concept that the major pathway of lipogenesis in adipose tissue is not different from pathways described for the liver. A small fraction of the propionate is converted to long-chain fatty acids in adipose tissue *in vitro* by a mechanism that involves the use of propionate as an intact three-carbon unit, but in any case, its significance in the intact animal would appear to be negligible.

The liver was long considered as the major, not the sole, site of lipogenesis in the mammalian organism (1). However, recently Favarger and Gerarch (2) and Favarger (3) have presented evidence that indicates that the liver does not play a major role in this process. These authors found that hepatic lipogenesis can account for only 4% of the total lipogenic activity of the mouse and that most of the remaining fatty acid synthetic activity is carried out in the adipose tissue.

In the last few years important advances in understanding the biochemical mechanism by which fatty acids are synthesized in the animal organism have been made (4, 5); however, most of these studies have been carried out on liver enzyme systems. It is of interest, therefore, that Feller and Feist (6, 7, 8) have presented considerable evidence indicating that adipose tissue can convert propionic acid as an intact three carbon unit into long-chain fatty acids, and have proposed a pathway of lipogenesis in adipose issue that is different from those described for liver (9). They have shown that this pathway does not occur in the liver, because in this organ the carboxyl carbon of propionate is not incorporated into long-chain fatty acids. If all of the above assumptions are true and the pathway proposed by Feller and Feist is a major one, it would be anticipated that most of the fatty acid synthesis in the intact animal would be found to occur via a pathway other than currently accepted ones. Since this pathway involves the use of propionic acid as a three-carbon unit, it would seem to be a corollary that administration of propionic acid labeled in the carboxyl position should lead to a large incorporation of the label into the body fatty acid.

METHODS

In Vivo Experiments. Male rats of the Wistar strain (inbred at Tufts for about 20 years) were kept at 25° for 10 days before the experiment and were allowed to eat the standard diet of the following composition: 25% casein, 10% lard, 50.95% glucose, 3% salt mix (10), 5% cellulose, 6% brewer's yeast, 0.04% cod liver oil concentrate, and 0.01% alpha tocopherol. The rats used in the experiments weighed 230 ± 10 g and two groups were fasted for 6 hours prior to the experiment. One group was given by stomach tube 1 g of triacetin plus 1.9 g dextrose containing a tracer dose of sodium acetate-1-C^{14}.[1] Another group was given 1 g of tripropionin plus 1.9 g dextrose containing sodium propionate-1-C^{14} in a tracer dose. The rats were then placed in a metabolism cage and their res-

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[1] The C^{14}-labeled compounds used in these experiments were purchased from the New England Nuclear Corporation, Boston, Mass.

piratory CO_2 was collected for 24 hours by the methods described in a previous paper (1). During the course of this metabolism study the rats had access to the diet described above.

The collected respiratory $C^{14}O_2$ was analyzed for its C^{14} content by a liquid counting method (11). At the end of the experiment the rats were killed by a blow on the head and the carcass was analyzed for lipids (12). The C^{14} content of the fatty acids was determined by pipetting 15 mg of the C^{14} fatty acids dissolved in $CHCl_3$ into a cupped nickel planchet and drying until the last trace of $CHCl_3$ had disappeared. The fatty acids were then counted at this mass and the counts were corrected to infinite thinness by a conversion factor. The C^{14} content of the labeled acetic acid and propionic acid was also measured (11).

A third group of rats was treated in a somewhat different manner. These rats were fed until the experiment was started. They were then injected intraperitoneally with 1 ml of saline containing either sodium acetate-1-C^{14} or sodium propionate-1-C^{14}. They were at once placed in wire cages and either fed or fasted. The respiratory $C^{14}O_2$ was not collected. The rats were sacrificed after 24 hours and the lipids isolated and analyzed from C^{14} content as described above.

In Vitro Experiments. Male rats of the above strain and male white Swiss mice (obtained from Mrs. Woodman, of Wayland, Mass.) were kept at 25° for 10 days on our standard diet. They were sacrificed by decapitation and the epididymal fat pad was excised and placed in a petri dish of Krebs-Henseleit bicarbonate buffer solution (13). A weighed piece of this adipose tissue (chosen randomly) was then placed into an incubation flask (14). The adipose tissue was kept at room temperature throughout this preincubation manipulation.

TABLE 1. METABOLISM OF GASTROINTESTINALLY-ADMINISTERED ACETATE-1-C^{14} AND PROPIONATE-1-C^{14} BY RATS

No. Rats Used*	Labeled Substrate†	Per Cent Labeled Substrate Converted to	
		CO_2	Fatty Acids
5	Acetate-1-C^{14}	78.8 ± 2.47‡	5.9 ± 0.75
5	Propionate-1-C^{14}	79.5 ± 7.07	0.3 ± 0.08

* Rats weighed 230. ± 10 g.

† 1 g of triacetin, 1.9 g of glucose and tracer dose of sodium acetate-1-C^{14} or 1 g of tripropionin, 1.9 g glucose and tracer dose of sodium propionate-1-C^{14} were administered by stomach tube; the rats were killed 24 hours later.

‡ S. E. (Note that in the case of the CO_2 analysis, the average value refers to only 4 rats.)

The main compartment of the incubation flask contained the adipose tissue, 4.5 ml of Krebs-Henseleit bicarbonate buffer containing glucose, 100 mg/100 ml, and 0.5 ml of saline containing 5 μmoles of the labeled substrate.. The system was gassed for about 30 seconds with 95% O_2-5% CO_2 gas mixture, sealed and incubated for 3 hours at 37.5° with continuous shaking.

The $C^{14}O_2$ formed during the incubation was recovered by the method of Chernick *et al.* (15) and its C^{14} content was then determined (11).

At the end of the incubation the adipose tissue was removed from the incubation flask and saponified in 30% KOH made up in a solvent of 50% ethanol. The fatty acids were then extracted from this hydrolyzate of adipose tissue by a method described earlier (16). The C^{14} content of the fatty acid was determined by a previously described method (17).

The C^{14} content of the labeled acetic acid and propionic acid was then determined (11).

RESULTS

The results of experiments in which rats were given by stomach tube either (*a*) sodium acetate-1-C^{14}, triacetin, glucose, or (*b*) sodium propionate-1-C^{14}, tripropionin, glucose, are recorded in Table 1. More than 75% of the C^{14} content of each of the labeled compounds appeared in the respiratory CO_2 during the 24 hours of the experiment. Significant quantities of C^{14} from the acetate-1-C^{14} appeared in the long-chain fatty acids of the body while C^{14} from propionate-1-C^{14} was almost completely absent. These results were surprising in light of the *in vitro* mouse experiments of Feller and Feist (6 to 9), and it seemed possible that these findings were due to the trapping by the liver of the labeled propionate entering it from the portal vein, thus preventing the propionate from reaching the adipose tissue. To circumvent this possibility to some extent, carboxyl-labeled propionate was administered intraperitoneally (Table 2), and the rats were then either fed or fasted for 24 hours. Very little of the C^{14} of the administered propionate-1-C^{14} appeared in the body's fatty acids. In a recently published investigation (18) Feller reported that about 2% of the C^{14} of intraperitoneally administered carboxyl-labeled propionate was found in the body fatty acids of mice fasted for 24 hours following the isotope administration.

Since the results of our *in vivo* studies are not consistent with the *in vitro* results of Feller and Feist (6 to 9), it was decided that the *in vitro* system should be reinvestigated. As shown in Table 3, rat liver is able to convert large amounts of acetate-1-C^{14} into fatty

TABLE 2. METABOLISM OF INTRAPERITONEALLY-ADMINISTERED PROPIONATE-1-C^{14} BY RATS

Nutritional State of the Rat Following Propionate-1-C^{14} Administration*	Per Cent Propionate-1-C^{14} Converted to Fatty Acids
Fed	0.5
Fed	0.3
Fasted	0.2
Fasted	0.1

* Rats weighed 230 ± 10 g; propionate-1-C^{14} was administered intraperitoneally in a tracer dose and the rats were killed 24 hours later.

acids but is unable to convert any of the carboxyl of propionate into long-chain fatty acids. On the other hand, while adipose tissue uses considerable amounts of the carboxyl carbon of acetate for lipogenesis, it uses very little of the carboxyl carbon of propionate for the purpose.

This finding led to an investigation of the conversion of propionate-1-C^{14}, propionate-2-C^{14}, and propionate-3-C^{14} to long-chain fatty acids by surviving adipose tissue (Table 4). Propionate-2-C^{14} and propionate-3-C^{14} entered fatty acids to a considerable extent, and at practically the same rate. In contrast, the conversion of propionate-1-C^{14} was very small. Since these results are not in agreement with those of Feller and Feist (7) in their studies with mice, propionate-1-C^{14} and propionate-2-C^{14} metabolism experiments were carried out on mouse adipose tissue (Table 5). It can be seen that in the mouse, as in the rat, propionate-2-C^{14} appears to be a much better substrate than propionate-1-C^{14} for lipogenesis.

TABLE 3. ACETATE-1-C^{14} AND PROPIONATE-1-C^{14} METABOLISM BY SURVIVING RAT LIVER SLICES AND ADIPOSE TISSUE

No. Rats Used*	Labeled Substrate†	Tissue Used‡	Per Cent C^{14} Converted to	
			CO_2	Fatty Acids
5	Acetate-1-C^{14}	Liver	33.7 ± 2.61§	4.8 ± 1.51
	Propionate-1-C^{14}	Liver	66.2 ± 1.46	0.0 ± 0.0
	Acetate-1-C^{14}	Adipose tissue	5.8 ± 0.32	4.0 ± 1.12
	Propionate-1-C^{14}	Adipose tissue	14.8 ± 0.77	0.6 ± 0.09

* Each substrate was incubated with liver and adipose tissue from each of the five rats; therefore results are a comparison of incubation flasks, not a comparison of different rats.
† The incubation system contained the tissue in 5 ml of Krebs-Ringer buffer containing 100 mg/100 ml glucose and 5 μmoles of labeled substrate; system was incubated at 37.5° for 3 hours.
‡ 250 mg of tissue used.
§ S. E.

The incubation medium used by Feller and Feist differs from ours in two particulars, namely, it contains more glucose than our medium and it contains 0.01 M succinate while our medium contains none. Therefore experiments using the succinate-containing medium were carried out (Tables 6 and 7). Although the results are not quantitatively identical to those found in our medium, it can be seen that propionate-2-C^{14} is still a better precursor of long-chain fatty acids than is propionate-1-C^{14}. It should be noted that the succinate depressed propionate metabolism in the case of rat adipose tissue, but that it promoted mouse adipose tissue lipogenesis, while still not abolishing the difference in the behavior of the 1 and 2 carbons.

TABLE 4. PROPIONATE METABOLISM BY SURVIVING RAT ADIPOSE TISSUE

No. Rats Used*	Labeled Substrate†	Per Cent‡ C^{14} Converted to	
		CO_2	Fatty Acids
5	Propionate-1-C^{14}	19.9 ± 3.06§	0.9 ± 0.25
	Propionate-2-C^{14}	7.0 ± 0.94	7.5 ± 1.50
	Propionate-3-C^{14}	7.0 ± 0.88	7.6 ± 1.50

* Each substrate was incubated with adipose tissue from each of the five animals; therefore results are a comparison of incubation flasks, not a comparison of different animals.
† See footnote, Table 3.
‡ 250 mg of adipose tissue were used in each flask.
§ S. E.

DISCUSSION

The experiments presented above indicate that the

TABLE 5. PROPIONATE METABOLISM BY SURVIVING MOUSE ADIPOSE TISSUE

No. Mice Used*	Labeled Substrate†	Per Cent‡ C^{14} Converted to	
		CO_2	Fatty Acids
5	Propionate-1-C^{14}	17.6 ± 1.46§	1.7 ± 0.32
	Propionate-2-C^{14}	6.7 ± 0.58	7.3 ± 0.61

* See footnote, Table 4.
† See footnote, Table 3.
‡ 200 mg of adipose tissue used in each flask.
§ S. E.

TABLE 6. PROPIONATE METABOLISM BY SURVIVING RAT ADIPOSE TISSUE INCUBATED IN A SUCCINATE-CONTAINING MEDIUM

No. Rats Used*	Labeled Substrate†	Per Cent‡ C^{14} Converted to	
		CO_2	Fatty Acids
5	Propionate-1-C^{14}	7.2 ± 0.30§	0.6 ± 0.06
	Propionate-2-C^{14}	3.3 ± 0.30	2.3 ± 0.35

* See footnote, Table 4.

† The incubation system contained the tissue in 5 ml of the incubation medium of Feller and Feist (7) containing 5 μmoles of the labeled substrate. The system was incubated at 37.5° for 3 hours.

‡ 250 mg of tissue were used.

§ S. E. (Note that the value of 7.2 refers to only 4 rats.)

carboxyl carbon of propionate is not used to an appreciable extent in the biosynthesis of long-chain fatty acids in the intact rat. As a corollary of this finding, it can be concluded that in the intact organism a very small fraction of fatty acid synthesis occurs via a pathway utilizing propionate as an intact molecule. This is true whether the propionic acid is ingested or is administered to the animal by intraperitoneal injection.

In the case of surviving adipose tissue, it is evident that the carboxyl carbon of acetate is far more readily incorporated into long-chain fatty acids than is the carboxyl label of propionate. Furthermore, on the basis of varying the position in which propionate is labeled, it is evident that propionate-2 or -3 carbons are far better precursors of long-chain fatty acids than is the carboxyl carbon. It seems evident, therefore, that even in adipose tissue studied *in vitro*, most of fatty acid synthesis is not occurring via a pathway that involves the conversion of the propionate molecule as a unit into long-chain fatty acids, but rather by pathways not inconsistent with those described for the liver by Brady (4) and Wakil *et al.* (5).

TABLE 7. PROPIONATE METABOLISM BY SURVIVING MOUSE ADIPOSE TISSUE INCUBATED IN A SUCCINATE-CONTAINING MEDIUM

No. Mice Used*	Labeled Substrate†	Per Cent‡ C^{14} Converted to	
		CO_2	Fatty Acids
5	Propionate-1-C^{14}	15.7 ± 1.26§	3.1 ± 0.60
	Propionate-2-C^{14}	4.4 ± 0.10§	15.5 ± 1.85

* See footnote, Table 4.

† See footnote, Table 6.

‡ 100 or 150 mg of tissue used; all values are corrected to that of 100 mg of tissue.

§ S. E. (Note that the value of 4.4 refers to only 4 mice.)

It will be clear that some of the data presented in this paper lead to conclusions which are at variance with those advanced by Feller and Feist (7, 8, 18). In the case of the *in vivo* experiments, the possibility existed that the variance could be attributed to species difference since Feller (18) used mice and we used rats. However, in the case of the *in vitro* experiments, similar results were obtained by us with mice as with rats. It is true that minor differences between our technique and those of Feller and Feist exist, such as the composition of the diet, the region of the body from which the fat was taken, and the method of slicing and chilling the tissue. These differences, however, were not of a nature, it seems to us, to explain the discrepancies in our results.

Our data with propionate-1-C^{14} indicate that in isolated adipose tissue a small fraction of the propionate enters long-chain fatty acids by a mechanism involving the use of propionate as an intact three-carbon unit. This finding is compatible with the possibility that the pathway suggested by Feller and Feist (9) is operative to a limited extent in adipose tissue. However, it seems probable from our data that such a pathway has little or no activity in the intact organism.

Since the submission of this manuscript for publication, Favarger and Gerlach (19) have published a report of their studies on the synthesis of fatty acids from propionate by the intact mouse. They find that three times as much C^{14} is incorporated into fatty acids when propionate-2-C^{14} is administered intravenously to mice than when propionate-1-C^{14} is used. Propionate-1-C^{14} is primarily incorporated in the odd-chain fatty acids and on basis of degradation studies the authors have concluded that intact propionate molecules are incorporated in the ω position of odd-chain acids and that the 2 and 3 carbons of propionate are incorporated via acetyl-CoA throughout the carbon skeleton of odd- and even-chain fatty acids. Our results are completely compatible with the findings and conclusions of Favarger and Gerlach.

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UTILIZATION OF RUMEN METABOLITES AS ENERGY SOURCES IN RATS[1]

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Acetate, propionate and butyrate have been well established as end products of carbohydrate digestion in the rumen (1). Concern for the efficiency of utilization of these compounds as energy sources by the ruminant was stimulated by the work of Armstrong and Blaxter (2, 3). In a previous report from this laboratory (4) acetate, propionate and butyrate were digested as well as glucose and starch in mature rats. That work suggested a possible use of monogastric laboratory species to study the efficiency of rumen metabolite utilization. This study was initiated to compare the utilization of energy from acetate, propionate or butyrate with glucose and starch in weanling and adult rats.

Experimental

The compositions of the diets used in these experiments are given in Table 1. The diets were formulated to vary only in the source of 24% of the gross energy. Acetate, propionate and butyrate in the form of triglycerides were substituted on a caloric basis for one-half the glucose and starch of the control diet. Glycerol content was equalized to compensate for the glycerol in the triglycerides. Females rats, initially weighing approximately 50 g, were used in experiment I and older rats weighing approximately 140 g were used in experiment II. The diets were offered daily at levels to provide [illegible] kcal gross energy per g body weight$^{0.75}$ for the 50 g rats and 0.75 kcal

[1] The investigation reported in this paper (No. 68-5-33) is in connection with a project of the Kentucky Agricultural Experiment Station and is published with the approval of the Director.

TABLE 1

Composition of Rations Fed to Rats in Energy Experiments (grams)

	Control	Acetate	Propionate	Butyrate
Soybean protein	15.00	15.00	15.00	15.00
Casein	10.00	10.00	10.00	10.00
Corn oil	5.00	5.00	5.00	5.00
Mineral mix[1]	5.00	5.00	5.00	5.00
Vitamin mix[2]	5.00	5.00	5.00	5.00
Cellulose	2.00	2.00	2.00	2.00
Starch	21.00	9.13	9.13	9.13
Glucose	22.47	9.21	9.21	9.21
Glycerol	14.53	-----	6.24	8.73
Triacetin	-----	34.42	-----	-----
Tripropionin	-----	-----	23.43	-----
Tributyrin	-----	-----	-----	19.03
Total grams	100.00	95.76	90.01	88.10
Gross energy (kcal/g) analyzed	4.10	4.47	4.66	4.70

[1] Formulated after: Wesson's modification of Osborne and Mendel's salt mixture (Science 75:339. 1932).

[2] Composition: (in g/100g) choline chloride, 3.3039; inositol, 0.2202; a-tocopherol, 0.2202; niacin, 0.1982; Ca pantothenate, 0.1321; menadione, 0.0995; riboflavin, 0.0440, pyridoxine HCl, 0.0440; thiamine HCl, 0.0440; (in mg/100g) folic acid, 3.9647; biotin, 0.8810; vitamin B_{12}, 0.6800; (in I.U/100g) vitamin A, 39,645; vitamin D, 4.956; remainder starch.

per g body weight$^{0.75}$ for the 140 g rats. The animals were weighed and feed allowances adjusted at 5-day intervals. Refused feed was removed and weighed daily.

For the determination of digestible and metabolizable energy of these rations, rats were confined to conventional metabolism cages designed for

Energy

opionate	Butyrate
15.00	15.00
10.00	10.00
5.00	5.00
5.00	5.00
5.00	5.00
2.00	2.00
9.13	9.13
9.21	9.21
6.24	8.73
-----	-----
23.43	-----
-----	19.03
90.01	88.10
4.66	4.70

rne and Mendel's salt

39; inositol, 0.2202;
enate, 0.1321;
e HCl, 0.0440;
.9647; biotin, 0.8810;
,645; vitamin D, 4.956;

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was removed and weighed

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a cages designed for

separate collections of feces and urine. Following a 10-day preliminary period, total collections of feces and urine were made for 5 days. Samples of rations, feces and urine were analyzed for gross energy with an oxygen bomb calorimeter.

Rats were randomly assigned to 5 groups to be used in the growth and energy balance experiments. One group was slaughtered initially to determine initial carcass composition and the remaining groups were fed the experimental rations for 20 days and slaughtered to obtain growth and final carcass composition data. Sacrifice of rats and carcass analyses were by the procedure of Hartsook and Hershberger (5). All data were subjected to analysis of variance and differences among means assessed for significance by the method of Duncan (6).

Results and Discussion

Feeding of the propionate ration to the 50 g rats (experiment I) was discontinued after 5 days owing to high mortality and negative weight gains. All older rats survived (experiment II), but rough hair coats, reddening of the paws and extreme excitability were observed in the rats fed propionate.

Digestible and metabolizable energy values are presented in Table 2.

TABLE 2

Digestible and Metabolizable Energy of Rations Containing Acetate, Propionate, or Butyrate Substituted for Glucose and Starch. (% of ration gross energy)

	Control	Acetate	Propionate	Butyrate
Experiment I				
Number of rats	6	6	6	6
Digestible energy	94.8	95.5	-----	94.8
Metabolizable energy	92.7	93.7	-----	93.1
Experiment II				
Number of rats	4	4	4	4
Digestible energy	93.9	93.2	94.1	93.5
Metabolizable energy	90.4	89.5	90.4	90.4

TABLE 3

Utilization of Energy of Rat Rations Containing Acetate, Propionate, or Butyrate Substituted for Starch and Glucose

	Experiment I			Experiment II			
	Control	Acetate	Butyrate	Control	Acetate	Propionate	Butyrate
Number of rats	9	9	9	7	7	7	7
Intake kcal M.E.	466.8	449.7	466.3	795.6	766.6	714.7	732.6
Initial wt, g	45.6	45.8	50.6	140.4	139.4	137.9	138.7
Gain, g	50.4	44.6	40.6[a]	32.9	31.7	33.0	23.9[a]
Kcal M.E./g gain	9.4	11.3[a]	11.8[a]	24.1	24.2	21.7	30.7
Carcass energy gain, kcal	92.2	84.6	93.5	113.1	99.5	100.3	69.0
Maintenance energy, kcal	238.4	231.8	238.7	447.5	431.5	429.9	417.1
Net energy, kcal	330.6	316.4	332.2	560.6	531.0	530.2	486.1
Net energy, % of metabolizable	71.5	71.3	72.0	69.9	72.2	74.2	67.9

[a] Significantly different from control ($P<.05$)

Net energy, kcal	330.6	316.4	332.2	560.6	531.0	530.2	486.1	
Net energy, % of metabolizable	71.5	71.3	72.0	69.9	72.2	74.2	67.9	

[a] Significantly different from control (P<.05)

The energy of diets containing acetate, propionate or butyrate, in the form of triglycerides, substituted for starch and glucose, was digested and metabolized as well as the energy of the control diet. The digestible and metabolizable energy values were similar to those reported previously (4, 8).

The growth and body energy balance data are summarized in Table 3. In experiment I, rats fed the diet containing acetate had a lower intake of metabolizable energy and lower weight gains than did the controls. Body weight gains were also lower for rats fed the diet containing butyrate. These lower gains were accompanied by significant (P<.05) increases in metabolizable energy required per unit of weight gain.

Carcass energy gains were lower for rats fed the diet containing butyrate in experiment II. Energy expended for maintenance was calculated by adding a 25% increment to the basal metabolism calculated from average metabolic weight (7). Net energy values represent the sum of body energy gain and calculated energy expended for maintenance. There were no significant differences in the utilization of metabolizable energy.

Carcass dry matter compositions of rats fed these diets are shown in Table 4. While there were no significant differences in the energy content per gm or % crude protein, the carcasses of rats fed butyrate in experiment I contained more fat than did the carcasses of rats fed other diets. While highly variable in experiment II, fat content of the carcasses of rats fed the control diet tended to be higher than with the diets containing acetate, propionate or butyrate.

Blaxter (9) has attributed differences in efficiency of energy utilization in ruminants of short chain acids to differences in heat increment. Such differences would not be reflected in determinations of digestible and metabolizable energy but would be expected to affect net energy values. While differences were noted in weight gains and some carcass constituents appeared to vary, no consistant differences were found in the utilization of metabolizable energy of these rations as indicated by the net energy values.

TABLE 4

Composition of Carcass Dry Matter of Rats Fed Rations Containing Acetate, Propionate, or Butyrate Substituted for Starch and Glucose

	Control	Acetate	Propionate	Butyrate
Experiment I				
Number of rats	9	9	-----	9
Kcal/g	5.74	5.78	-----	5.97
Crude protein, %	50.2	50.6	-----	50.6
Ether extract, %	29.2	31.3	-----	37.9[a]
Experiment II				
Number of rats	7	7	7	7
Kcal/g	6.46	6.33	6.24	6.07
Crude protein, %	55.6	50.0	47.3	46.9
Ether extract, %	32.1	23.3	18.5	20.0

[a] Significantly different from control ($P<.05$).

This suggests that the energy of acetate, propionate and butyrate was utilized as efficiently as the glucose and starch for which they were substituted.

The apparent metabolic problems observed in rats fed propionate were not investigated further in this study; however, dietary propionate has been reported by others to induce metabolic disturbances in rats which can be partially alleviated with additional vitamin B_{12} in the diet (10, 11, 12, 13). Gurnani et al. (14) have described the role of vitamin B_{12} in isomerization of methylmalonyl-Co A to succinyl-Co A in propionate metabolism, and this appears to be a limiting reaction in propionate metabolism in rats fed diets either adequate or deficient in vitamin B_{12} (12, 13). Diets used in this experiment were heavily fortified with vitamin B_{12}. A clear relationship has not been established between propionate metabolism and stress on the supply of other B vitamins.

Summary

The utilization of the energy from acetate, propionate, and butyrate when providing 24% of the dietary gross energy was studied in female rats weighing approximately 50 and 140 g. Substitution of acetate, propionate, or butyrate for starch and glucose did not significantly affect the digestible, metabolizable, or net energy measurements. Dietary propionate appeared to interfere with normal metabolic function to the extent that this diet could not be fed to the younger rats. While older rats did have rough hair coats, reddening of the paws, and were extremely excitable, their performance was not different from that of rats receiving the control ration when measured by the parameters used in this experiment.

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uing oils; waterproofing fabrics as co[illegible]ion inhibitor in halo

Pantothenic acid calcium salt; rdroxy-3,3-dimethylbutyryl)-[illegible] C(CH₃)₂CHOHCONHCH₂CH₂ $C_{18}H_{32}CaN_2O_{10}$; C 45.37 .88%, O 33.57%. Prepn: Wehr; 5 (1957 to Commercial Solvents 2,846,456 (1958 to Upjohn n, U.S. pat. 2,935,528 (1960 to Monograph: Greulich, Meiner. *See also* Pantothenic Acid for Only the dextrorotatory isomer activity.

nethanol. Sweetish taste with Dec 195–196°. Moderately hy- ble to air and light. $[\alpha]_D^{23}$ +28.2 ; one gram dissolves in 2.8 ml ily sol in alcohol, acetone. pH een 7.2 and 8.0; pH in CO_2-free ntothenate solns are most stable of hydrolysis is a function of pH sence of electrolytes. Solns are and sterilization by filtration is Frost, McIntire, *J. Am. Chem.*

: Acid.
ntothenic acid.

$Ca(MnO_4)_2$; mol wt 277.94. 46.05%. Prepn from $KMnO_4$ 5 (1949 to Boots Pure Drug Co., om $Al(MnO_4)_3$ and $Ca(OH)_2$: .130 (1950 to Carus Chemical

liquesce crystals. Freely sol in *tightly closed.*
int, deodorizer; with CaF_2 as coatings and fluxes.

ium dioxide. CaO_2; mol wt 0%. The commercial product 0% = 13.3% available Ca((and $CaCO_3$. Prepn: (1950 to Du Pont); Ehrlich in *organic Chemistry*, G. Brauer, s, 1963), p 936.
iess, almost tasteless powder. sol in water; sol in acids with *ll closed.*

septic oxidizing agent.

p-Hydroxybenzenesulfonic acid polate; calcium sulfophenolate. wt 386.40. $C_{12}H_{10}CaO_8S_2$; 0.37%, O 33.12%, S 16.60%. n. *Praxis* vol. 2, 420 (Berlin,

$_2]_2.H_2O$, odorless cryst pow- ohol. The aq soln is neutral, ste.
as an intestinal antiseptic, in nd slowly granulating wounds

um carbolate; calcium phenate; phenylate. $Ca(OC_6H_5)_2$; mol 3.69%, H 4.45%, Ca 17.72%, Drake, U.S. pat. 2,870,134

air. Slightly sol in water or

: motor oils.

ic. Calcium monohydrogen phosphate. $CaHPO_4$; mol .74%, O 47.04%, P 22.77%. PO_4: Jensen, Rathley, *Inorg.* $_3(PO_4)_2$ and H_3PO_4: Perloff, re it is an intermediate in the

. Triclinic crystals. At red heat dehydrated to calcium pyrophosphate. Practically insol in water, alcohol.

Dihydrate, $CaHPO_4.2H_2O$, ***Brushite.*** Monoclinic crystals. Water of crystn lost slowly below 100°. Dehydrated at red heat to calcium pyrophosphate. d 2.31. Practically insol in water, alcohol; sol in dil HCl or HNO_3; slightly sol in dil acetic acid.

USE: Chiefly in animal feeds; mineral supplement in cereals and other foods; manuf of glass; in dental products, fertilizers (*see also* Calcium Phosphate, Monobasic).

MED USE: Dietary supplement. *Dose:* Oral 1 g.

VET USE: *See* Calcium Phosphate, Tribasic.

Calcium Phosphate, Monobasic. Acid calcium phosphate; calcium biphosphate; monocalcium phosphate; primary calcium phosphate; "calcium superphosphate." $CaH_4(PO_4)_2$; mol wt 234.06. Ca 17.12%, H 1.72%, O 54.69%, P 26.47%. Commercial prepn for fertilizers by treating pulverized phosphate rock with H_2SO_4 or H_3PO_4: Faith *et al.*, *Industrial Chemicals* (3rd ed, John Wiley & Sons, Inc., 1965), p 194. Laboratory prepn from $CaCO_3$ and H_3PO_4: Jensen, Kathley, *Inorg. Syn.* 4, 18 (1953).

Hydrate, $CaH_4(PO_4)_2.H_2O$, large, shining, triclinic plates, cryst powder or granules. Non-hygroscopic when pure, but traces of impurities such as H_3PO_4 cause material to be deliquesc. Strong acid taste. Loses H_2O at 100°, dec at 200°. d_4^{18} 2.220. Moderately sol in water; sol in dil HCl or HNO_3 or acetic acid.

Note: The products obtained from commercial processes are not pure monobasic calcium phosphate. The ***superphosphate*** obtained from the H_2SO_4 treatment is about 30% $CaH_4(PO_4)_2.H_2O$, 10% $CaHPO_4$, 45% $CaSO_4$, 10% iron oxide, silica, alumina, etc. and 5% water; it contains 18–21% available P_2O_5. The ***triple superphosphate*** obtained from the H_3PO_4 treatment contains from 43 to 50% available P_2O_5.

USE: Chiefly in fertilizers; as acidulant in baking powders and in wheat flours; mineral supplement for foods and feeds; in enameling.

Calcium Phosphate, Tribasic. Tricalcium phosphate; tertiary calcium phosphate. The technical product is also known as "bone ash." Calcigenol Simple. $Ca_3(PO_4)_2$; mol wt 310.20. Ca 38.76%, O 41.26%, P 19.97%. It is about 96% pure, usually contg an excess of CaO. Occurs in nature as the minerals: ***oxydapatit, voelicherite, whitlockite.*** Commercial prepn from phosphate rock: Hignett, Hubbard, *Ind. Eng. Chem.* 38, 1208 (1946). Elmore, U.S. pat. 2,474,831 (1949 to Tennessee Valley Authority); Hollingsworth, U.S. pats. 2,556,541 and 2,562,718 (both 1951 to Coronet Phosphate Co.); Brosheer, Hignett, *Tenn. Valley Authority, Chem. Eng. Rept.* no. 7, 143 pp (1953).

Amorphous, odorless, tasteless powder. d 3.14. mp 1670°. Practically insol in water, alcohol or acetic acid; sol in dil HCl or HNO_3.

USE: Manuf of fertilizers, H_3PO_4 and P compds; manuf milk-glass, polishing and dental powders, porcelains, pottery; enameling; clarifying sugar syrups; in animal feeds; as noncaking agent; in the textile industry.

MED USE: Gastric antacid. *Dose:* Oral 1 g.

VET USE: Antacid. As source of calcium, phosphorus. *Dose:* horses: 30–60 g; dogs: 2–10 g.

Calcium Phosphide. Photophor. Ca_3P_2; mol wt 182.20. Ca 65.99%, P 34.01%. Prepn: Ehrlich in *Handbook of Preparative Inorganic Chemistry*, G. Brauer, Ed., (2nd ed, Academic Press, 1963), p 942.

Red-brown cryst powder or gray lumps. Dec by moist air or water, evolving spontaneously-flammable phosphine. d 2.51; mp about 1600°. *Keep dry and tightly closed.*

USE: For signal fires; in purification of Cu and Cu alloys; as rodenticide.

Calcium Phosphite. $CaHPO_3$; mol wt 120.07. Ca 33.38%, H 0.84%, O 39.98%, P 25.80%. Prepn: *Gmelin's Handb. anorg. Chem.*, System no. 28 (Calcium), part B, 8th ed, p 1121 (1958).

Hydrate, $CaHPO_3.H_2O$, crystals. Loses water at 200°; dec above 300°. Slightly sol in water; practically insol in alcohol.

USE: Fertilizers; polymerization catalyst.

Calcium Propionate. ***Propionic acid calcium salt;*** Mycoban. $Ca(CH_3CH_2COO)_2$; mol wt 186.22. $C_6H_{10}CaO_4$; C 38.70%, H 5.41%, Ca 21.52%, O 34.37%. Occurs with $1H_2O$ or $3H_2O$. Prepn: *Beilstein* vol. 2, 238, 2nd suppl., 218, 3rd suppl., 516.

Powder or monoclinic crystals. Soluble in water; slightly sol in methanol, ethanol; practically insol in acetone, benzene.

USE: As an inhibitor of molds and other microorganisms in foods, tobacco, pharmaceuticals; in butyl rubber to improve processability and scorching resistance.

MED USE: Has been used as antifungal agent in dermatology.

Calcium Pyrophosphate. Calcium diphosphate. $Ca_2P_2O_7$; mol wt 254.12. Ca 31.54%, O 44.08%, P 24.38%. Prepn by ignition of $CaHPO_4$: St. Pierre, *J. Am. Chem. Soc.* 77, 2197 (1955).

Polymorphous crystals or powder. d 3.09. mp 1353°. Practically insol in water; sol in dil HCl or HNO_3.

USE: Abrasive; fertilizer; feed supplement; in dentifrices, ceramic ware, china, glass, phosphors.

Calcium D-Saccharate. ***D-Glucaric acid calcium complex.*** $CaC_6H_8O_8$; mol wt 248.21. C 29.03%, H 3.25%, Ca 16.15%, O 51.57%. The normal calcium salt of D-saccharic acid, a dicarboxylic sugar acid derived from the oxidation of D-gluconic acid. Calcium D-saccharate is a true chemical compd and should not be confused with saccharated lime, formerly called "calcium saccharate" and produced by the action of lime upon sugar. Prepn: *Beilstein*, vol. 3, 2nd suppl., 378; *Hagers Handb. pharm. Praxis* vol. 1, 755 (Berlin, 1930).

Tetrahydrate, $CaC_6H_8O_8.4H_2O$, odorless, tasteless crystals or fine white powder. Stable to air. Becomes anhydr upon heating at 100° *in vacuo.* Practically insol in water, alcohol, ether. Soluble in dil mineral acids and in calcium gluconate solns.

USE: As plasticizer in cement, concrete, mortar.

MED USE: Stabilizer for calcium gluconate solns for parenteral injection.

Calcium Salicylate. $Ca[C_6H_4(OH)COO]_2$; mol wt 314.30. $C_{14}H_{10}CaO_6$; C 53.50%, H 3.21%, Ca 12.75%, O 30.54%. Prepn: Coninck, *Rev. gen. chim.* 17, 72 (1914).

Dihydrate, $Ca(C_7H_5O_3)_2.2H_2O$, monoclinic crystals or powder. Odorless; tasteless. Loses all H_2O at 120°. Dec at 244° to phenol, the basic salicylate, CO_2 and H_2O. Soly in water at 15.5°: 28.46 g/l; soly in ethanol at 16.7°: 15.5 parts/l; slightly sol in methyl acetate; sol in methanol. The aq soln is slightly acid and light sensitive.

Calcium Selenate. $CaSeO_4$; mol wt 183.04. Ca 21.90%, O 34.97%, Se 43.14%. Prepd by treating a soln of calcium nitrate with potassium selenate: Mitscherlich, *Pogg. Ann.* 9, 623 (1827); von Hauer, *Sitzungsber. Akad. Wien* 39, 299, 839 (1860).

Dihydrate, $CaSeO_4.2H_2O$, monoclinic crystals. d_4^{20} 2.69. Gradually loses H_2O on heating becoming anhydr by 200°; dec to $CaSeO_3$ at 698°. Soluble in water.

USE: Pesticide.

Calcium Selenide. CaSe; mol wt 119.04. Ca 33.67%, Se 66.33%. Prepd by reducing $CaSeO_4$ in a stream of H_2 at 400–500°: Ehrlich in *Handbook of Preparative Inorganic Chemistry*, G. Brauer, Ed., (2nd ed, Academic Press, 1963), p 939.

White powder. In air may turn red within a few minutes and light brown in a few hours. d 3.82. Decompd by water. Treatment with HCl produces H_2Se gas, and red Se separates.

USE: In electron emitters.

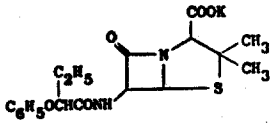
Calcium Silicate. Many different forms of calcium silicate are known. Among the most common forms are $CaSiO_3$, Ca_2SiO_4 and Ca_3SiO_5. Usually occur in hydrated form contg various percentages of water of crystallization. Names of calcium silicate minerals are: ***Afwillite; akermannite; calcium pectolith; centrallasite; crestmoreite; eaklite; foshagite; foshallasite; gjellebaekite; grammite; gyrolite; hillebrandite; larnite; okenite; parawollastonite; pseudo-wollastonite; riversideite; table spate; tobermorite; wollastonite; xonaltite; xonotlite.*** Commercial calcium silicate sold for industrial use, such as ***Micro-Cell*** and ***Silene,*** is prepared synthetically to control its absorbing power. The usual method of prepn is from lime and diatomaceous earth under carefully controlled conditions: Boss, *Chem. Eng. News* 27, 677 (1949); Steinour, *Chem. Revs.* 40, 391 (1947). The commercial product is described here.

White or slightly cream-colored, free-flowing powder. Approximate analysis: CaO 19%, SiO_2 67%, H_2O 6 to 8%.

propyl ester; isopropyl 1-methyl-4-phenylisonipecotate; ipropethidine; isopedine; Spasmodolisina; Gevelina. $C_{16}H_{23}NO_2$; mol wt 261.35. C 73.53%, H 8.87%, N 5.36%, O 12.24%. Prepn: Bergel *et al.*, *J. Chem. Soc.* **1944**, 265. Hydrochloride, $C_{16}H_{23}NO_2.HCl$, crystals, mp 192–195°. Soluble in water.

MED USE: The hydrochloride as analgesic, antispasmodic.

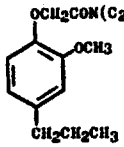
Propicillin. *3,3-Dimethyl-7-oxo-6-(2-phenoxybutyramido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid potassium salt;* α-phenoxypropyl penicillin potassium; 6-(α-phenoxybutyramido)penicillanic acid potassium salt; potassium α-phenoxypropylpenicillin; levopropylcillin potassium; Baycillin; Brocillin; Cetacillin; Oricillin; Trescillin; Ultrapen; BRL 284; PA-248. $C_{18}H_{23}KN_2O_5S$; mol wt 416.55. C 51.90%, H 5.08%, K 9.39%, N 6.73%, O 19.21%, S 7.70%. Prepn from 6-aminopenicillanic acid: Perron *et al.*, *J. Am. Chem. Soc.* **82**, 3934 (1960); Brit. pat. 877,120 (1961 to Beecham Research Laboratories); Glombitza, *Ann.* **673**, 166 (1964).



Crystals, dec 195–197°. Soluble at 20° in 1.2 parts water, 23 parts 95% (w/v) alcohol. pH of 1% w/v soln: 5–7.5.

MED USE: Antimicrobial. *Dose:* Oral 125–500 mg.

Propinal. *N,N-Diethyl-2-(2-methoxy-4-propylphenoxy)-acetamide;* 2-methoxy-4-propylphenoxyacetic acid *N,N*-diethylamide; E-34; 2-M-4-P. $C_{16}H_{25}NO_3$; mol wt 279.37. C 68.78%, H 9.02%, N 5.01%, O 17.18%. Prepn: Brit. pat. 810,539 (1959 to J. R. Geigy).



Viscous oil, $bp_{0.001}$ 121–123°.

MED USE: i.v. anesthetic.

β-Propiolactone. *Hydracrylic acid β-lactone;* Betaprone; β-propionolactone; 2-oxetanone; propanolide. $C_3H_4O_2$; mol wt 72.06. C 50.00%, H 5.60%, O 44.41%. Prepd by the condensation of ketene with formaldehyde in the presence of zinc or aluminum chloride: Küng, U.S. pat. 2,356,459 (1941). Purification: Gresham, Jansen, U.S. pat. 2,602,802 (1952 to B. F. Goodrich Co.).

Liquid. d_4^{20} 1.1460; d_4^{25} 1.1420. d_{20}^{20} 1.1490. mp −33.4°. bp_{760} 162° (decompn); bp_{760} 150° (decompn); bp_{20} 61°; bp_{10} 51°. Flash pt 70° (158°F). n_D^{20} 1.4131; n_D^{25} 1.4110. Dipole moment 3.8. Slowly hydrolyzed to hydracrylic acid. Stable when stored in ice box (+5°) in glass containers. Solubility in water: 37% v/v. Miscible with alcohol (reaction), acetone, ether, chloroform. LD_{50} i.v. in mice: 345 mg/kg.

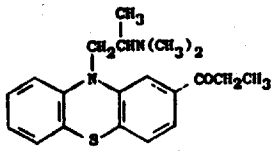
USE: Versatile intermediate in organic synthesis, *e.g.*, in place of acrylic acid in the Diels-Alder diene synthesis: Gresham, Jansen, Werber, *J. Am. Chem. Soc.* **76**, 609 (1954).

MED USE: To sterilize vaccines, grafts, plasma.

Propiolic Acid. Acetylenecarboxylic acid; propargylic acid; propynoic acid. CH≡CCOOH; mol wt 70.05. $C_3H_2O_2$; C 51.44%, H 2.88%, O 45.68%. Prepn: Wilson, Wenzke, *J. Am. Chem. Soc.* **57**, 1265 (1935); Owen, Sultanbawa, *J. Chem. Soc.* **1949**, 3109; Wolf, *Ber.* **86**, 735 (1953), **87**, 668 (1954). Manuf: Wolf, U.S. pat. 2,786,022 (1957); Pachter, U.S. pat. 2,799,703 (1957 to Ethyl Corp.). *Review* on its occurrence and biological activity: Reisch, *Pharmazie* **20**, 194 (1965).

Liquid at room temp (crystals from carbon disulfide, mp 9°). bp 144° (dec), $bp_{10.5}$ 54–55°, bp_{50} 70–75°. d_4^{15} 1.1435, d_4^{20} 1.1380, d_4^{25} 1.1325. $n_D^{20.4}$ 1.4302. Dipole moment at 25°, 2.08D (in dioxane): Wilson, Wenzke, *loc. cit.*

Propiomazine. *1-[10-(2-Dimethylaminopropyl)phenothiazin-2-yl]-1-propanone;* 10-(2-dimethylaminopropyl)-2-propionylphenothiazine; propionylpromethazine; 3-propionyl-10-dimethylaminoisopropylphenothiazine; 10-dimethylaminoisopropyl-2-propionylphenothiazine; Phenoctyl; Wy-1359. $C_{20}H_{24}N_2OS$; mol wt 340.50. C 70.55%, H 7.10%, N 8.23%, O 4.70%, S 9.42%. Prepn: Schmitt *et al.*, *Bull. Soc. Chim. France* **1957**, 1474; Schmitt, Fr. addn. 71,342 (addn. to Fr. pat. 1,176,919 to Clin-Byla).



Maleate, $C_{20}H_{24}N_2OS.C_4H_4O_4$, *Dorevane, Indorm, Largon, Propavan, 1678 CB.* Crystals from isopropanol, mp 160–161° (base, $bp_{0.5}$ 235–245°). LD_{50} in mice: 288 mg/kg. Also used as the hydrochloride, $C_{20}H_{24}N_2OS.HCl$.

Oxime, mp 173–174°.

Methiodide, mp 240–242°.

MED USE: Tranquilizer, hypnotic.

Propionaldehyde. *Propanal;* methylacetaldehyde; propylaldehyde. CH_3CH_2CHO; mol wt 58.08. C_3H_6O; C 62.04%, H 10.41%, O 27.55%. Prepd by treating propyl alcohol with a bichromate oxidizing mixture: Hurd, Meinert, *Org. Syn.* **12**, 64 (1932); by passing propyl alcohol vapor over copper at high temp: Sabatier, Senderens, *Compt. Rend.* **136**, 923 (1903); Willimott, *Analyst* **50**, 13; *Chem. Zentr.* **1925**, I, 2097.

Liquid. Suffocating odor. d_4^{0} 0.8432; $d_4^{9.7}$ 0.8192; d_4^{20} 0.8071; d_4^{25} 0.7898. mp −81°. bp_{760} 49°; bp_{740} 47°; bp_{737} 45°. $n_{656.6}^{18.6}$ 1.3695; n_D^{19} 1.36460. Absorption spectrum: Kwiecinski, Marchlewski, *Bull. Soc. Chim.* [4] **45**, 608 (1929). Soluble in 5 vols water at 20°. Miscible with alcohol and ether. LD_{50} orally in rats: 1.4 g/kg; lethal concn for rats in air: 8000 ppm.

Oxime, *propionaldoxime,* $CH_3CH_2CH{=}NOH$, needles, mp 40° [21.5°]; bp_{760} 131°; bp_{100} 77°; n_D^{20} 1.42938.

Human Toxicity: May cause respiratory irritation. *See* Acetaldehyde.

Propionamide. Propanamide; propionic acid amide. $CH_3CH_2CONH_2$; mol wt 73.09. C_3H_7NO; C 49.30%, H 9.65%, N 19.17%, O 21.89%. Prepd by heating ammonium propionate under pressure: Hofmann, *Ber.* **15**, 981 (1882); by dropping propionyl chloride into cooled ammonia water: Aschan, *Ber.* **31**, 2347 (1898).

Orthorhombic platelets from benzene. d_4^{20} 1.0335; d_4^{80} 0.9597; mp 79°; bp 222.2°. Volatile with steam. n_D^{120} 1.4160. Freely sol in water, alcohol, ether, chloroform. Lethal concn for rats in air: 8000 ppm.

Propionic Acid. Propanoic acid; methylacetic acid; ethylformic acid. CH_3CH_2COOH; mol wt 74.08. $C_3H_6O_2$; C 48.64%, H 8.16%, O 43.20%. Occurs in dairy products in small amounts. Can be obtained from wood pulp waste liquor by a fermentation process using bacteria of the genus *Propionibacterium:* Wayman *et al.*, U.S. pat. 3,067,107 (1962 to Columbia Cellulose Co., Ltd.). Prepn from ethylene, carbon monoxide, and steam: Reppe, *Angew. Chemie* **1956**, 46; Larson, U.S. pat. 2,448,375 (1948 to Du Pont); from ethanol and carbon monoxide using a boron trifluoride catalyst: Loder, U.S. pats. 2,135,448; 2,135,451; 2,135,453 (1939 to Du Pont); by oxidation of propionaldehyde: Hasche, U.S. pat. 2,294,984 (1942 to Eastman Kodak Co.); from natural gas by the Fischer-Tropsch process; as a byproduct in the pyrolysis of wood; by the action of microorganisms on a variety of materials in small yields. Very pure propionic acid can be obtained from propionitrile.

Oily liquid. Slightly pungent, disagreeable, rancid odor. d_4^{15} 0.99874; d_4^{20} 0.99336; d_4^{30} 0.98260. mp −21.5°. bp_{760} 141.1°; bp_{400} 122.0°; bp_{200} 102.5°; bp_{100} 85.8°; bp_{60} 74.1°; bp_{40} 65.8°; bp_{20} 52.0°; bp_{10} 39.7°; bp_5 28.0°; $bp_{1.0}$ 4.6°. n_D^{25} 1.3848. Viscosity in centipoises at 15° = 1.175; at 25°

= 1.020; at 30° = 0.956; at 40° = 0.841; at 60° = 0.668; at 80° = 0.544; at 90° = 0.495. Surface tension in dynes/cm at 15° = 27.21; at 20° = 339.5; critical press. 53.0 atm; critical density 0.315 g/ml. Sp heat between 20° and 137° = 0.560. Heat of fusion = 23.4 cal/g. Latent heat of vaporization = 98.8 cal/g; heat of combustion at 20° = 367 kcal/mole, also reported as 4,931.5 cal/g at 15°. Ka at 25° = 1.34×10^{-5}. Miscible with water. Can be salted out of water solns by the addition of calcium chloride or other salts. Soluble in alcohol, ether, chloroform. Azeotrope with water bp 99.98°, contains 17.7% acid; with toluene bp 110.45°, contains 3% acid; with *o*-xylene bp 135.4°, contains 43% acid; with ethylbenzene bp 131.1°, contains 28% acid.

Barium salt, $C_6H_{10}BaO_4.H_2O$, ***barium propionate***. Powder, usually with slight odor. *Poisonous!* Freely sol in water; slightly sol in alcohol.

USE: Esterifying agent; in the production of cellulose propionate (thermoplastic) and other propionates, *e.g.*, calcium propionate, used as mold inhibitors and preservatives; in the manuf of ester solvents, fruit flavors, and perfume bases. β-Propiolactone is useful in organic syntheses and to preserve arterial grafts.

MED USE: Salts have been used as antifungal agents in dermatology.

Propionic Anhydride. Propanoic anhydride; methylacetic anhydride. $(CH_3CH_2CO)_2O$; mol wt 130.14. $C_6H_{10}O_3$; C 55.37%, H 7.75%, O 36.88%. Obtained by dehydration of the acid or by carbonylation of its esters: Reppe, Friederich, U.S. pat. 2,730,546 (1956 to Badische Anilin- & Soda-Fabrik); from propionaldehyde by air oxidation in the presence of cobalt and copper acetate catalysts: McFarlane, U.S. pat. 2,491,572 (1949 to Celanese Corp.). Other syntheses, *e.g.*, from ethanol and carbon monoxide.

Liquid. Odor more pungent than that of the acid. d_4^0 1.0336; d_4^{15} 1.0169; d_D^{20} 1.0125; d_4^{40} 0.98974; d_4^{50} 0.97913. 8.4 lbs/gal at 20°. mp −45°. bp_{760} 167.0°; bp_{400} 146.0°; bp_{200} 127.8°; bp_{100} 107.2°; bp_{50} 94.5°; bp_{40} 85.6°; bp_{20} 70.4°; bp_{10} 57.7°; bp_5 45.3°; $bp_{1.0}$ 20.6°. Flash pt 74° (165°F). n_D^{17} 1.4041; n_D^{20} 1.4038. Viscosity in centipoises = 1.144 at 20°; 0.978 at 30°; 0.853 at 40°; 0.7511 at 50°. Dec by water. Soluble in methanol, ethanol, ether, chloroform. *p*-Toluidide deriv, mp 124°.

USE: Esterifying agent for certain perfume oils, fats, oils, and especially cellulose. In the production of alkyd resins, dyestuffs, and drugs. Has been used as a dehydrating agent in some sulfonations and nitrations.

Propionitrile. Propanenitrile; ethyl cyanide. CH_3CH_2CN; mol wt 55.08. C_3H_5N; C 65.42%, H 9.15%, N 25.43%, CN 47.24%, C_2H_5 52.76%. May be prepd by dehydration of propionamide (or propionic acid + NH_3) or by distilling ethyl sulfate and concd aq KCN, also by reduction of acrylonitrile.

Liquid. *Poisonous when heated to decompn or on contact with acids.* Pleasant, ethereal, sweetish odor. d_4^0 0.8020; d_4^{20} 0.7818; d_4^{40} 0.7716; d_4^{60} 0.7515; $d_4^{70.2}$ 0.7291. mp −91.8°. bp_{760} 97.2°, bp_{400} 77.7°; bp_{200} 58.2°; bp_{100} 41.4°; bp_{60} 30.1°; bp_{40} 22.0°; bp_{20} +8.8°; bp_{10} −3.0°; bp_5 −13.6°; $bp_{1.0}$ −35.0°. n_D^{15} 1.36812; n_D^{20} 1.36585; n_D^{30} 1.36132. Solubility in water at 40° = 11.9 g/100 g H_2O; at 100° = 28 g/100 g H_2O. Miscible with alcohol, ether, dimethylformamide. LD_{50} orally in rats: 39 mg/kg.

Human Toxicity: See Hydrogen Cyanide.

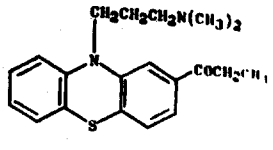
Propionyl Chloride. CH_3CH_2COCl; mol wt 92.53. C_3H_5ClO; C 38.94%, H 5.45%, O 17.29%, Cl 38.32%, propionic acid 80.06%.

Liquid, pungent odor. d_4^{20} 1.065; mp −94°; bp 80°; n_D^{20} 1.4051. Vigorously dec and dissolved by water or alcohol.

Propionylpromazine. *1-[10-[3-(Dimethylamino)propyl]-phenothiazin-2-yl]-1-propanone;* 3-propionyl-10-(γ-dimethylaminopropyl)phenothiazine; 10-(3-dimethylaminopropyl)-2-propionylphenothiazine; propiopromazine; 1497 CB; Combelen; Tranvex. $C_{20}H_{24}N_2OS$; mol wt 340.55. C 70.55%, H 7.10%, N 8.23%, O 4.70%, S 9.42%. Prepn: Schmitt *et al.*, *Bull. Soc. Chim. France* 1957, 938, 1474; Schmitt, Fr. addn. pat. 71,342 (1956 to Clin-Byla), *C.A.* 56, 3486h (1962).

Crystals, mp 69–70°.

Maleate, $C_{20}H_{24}N_2OS.C_4H_4O_4$, crystals from acetone, mp 135°.

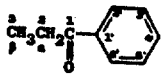


Oxime, $C_{20}H_{25}N_3OS$, pale yellow crystals, alcohol, mp 167–168°.

Methiodide, $C_{20}H_{24}N_2OS.CH_3I$, yellow [illegible] isopropanol, mp 79–80°.

Also used as hydrochloride, $C_{20}H_{24}N_2OS$ HCl.

Propiophenone. 1-Phenyl-1-propanone; ketone; propionylbenzene; phenyl ethyl ketone; mol wt 134.17. C 80.56%, H 7.51%, O 11.9[illegible] propionyl chloride and benzene in the presence aluminum chloride: Read, *J. Am. Chem. Soc.* 44, [illegible] Numerous other syntheses.



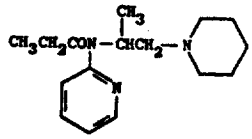
Leaflets or tabular crystals. Strong, persistent flowery odor. Usually supplied as a liquid. d_4^0 [illegible] d_4^{20} (liq) 1.0105; d_{20}^{20} 1.0118; d_{25}^{25} 1.0087; [illegible] $d_4^{51.8}$ 0.9776; $d_4^{86.8}$ 0.9572. mp 21°. Typical fr[illegible] 18.6°. bp_{760} 218.0°; bp_{400} 194.2°; bp_{200} [illegible] 149.3°; bp_{60} 135.0°; bp_{40} 124.3°; bp_{20} 107.6[illegible] bp_5 77.9°; bp_{10} 50.0°. $n_D^{15.9}$ 1.5290; n_D^{20} 1.5260. [illegible] max in hexane: 323; 280; 250 mμ. Dipole [illegible] Parachor 328.7. Flash pt 99° (210°F). Miscible [illegible] anol, anhydr ethanol, ether, benzene, toluene [illegible] water, glycerol, ethylene glycol, propylene glycol.

Oxime, $C_9H_{11}NO$, crystals, mp 54°, bp_{15} 14[illegible]

Semicarbazone, $C_{10}H_{13}N_3O$, crystals, mp 18[illegible] block).

USE: In perfumery; in the synthesis of epin[illegible] related compds.

Propiram. *N-(1-Methyl-2-piperidinoethyl)-N-[illegible] propionamide;* *N*-propionyl-2-(1-piperidinoso[illegible] pyridine. $C_{18}H_{25}N_3O$; mol wt 275.38. C 69.[illegible] N 15.26%, O 5.81%. Prepn: Fr. pat. M 2283 (1[illegible] AG.), *C.A.* 61, 645g (1964).

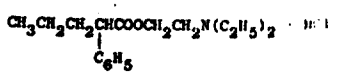


Free base, bp 162–163°.

Fumarate, $C_{18}H_{25}N_3O.C_4H_4O_4$, *FBA 4501.*

MED USE: Proposed as analgesic.

Propivane. Prospasmine; 177 R.P.; 2-diethyl[illegible] α-propyltoluate hydrochloride; *2-diethylaminoethyl valerate hydrochloride;* propylphenylacetate [illegible] aminoethanol hydrochloride. $C_{17}H_{27}NO_2$ HCl; 313.86. C 65.05%, H 8.67%, N 4.46%, O [illegible] 11.62%. Prepd by esterification of α-phenylval[illegible] diethylaminoethanol or by the action of diethyl[illegible] ester from α-phenylvaleric acid and glycol [illegible] drin: Viaud, U.S. pat. 2,219,796 (1941 to Rhône [illegible]

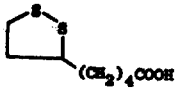


Crystals. Bitter taste. mp 109° (free base bp[illegible] Freely sol in water; very sparingly sol in alcohol. [illegible] 5% aq soln is neutral to litmus.

MED USE: Antispasmodic.

Propolis. Hive dross. Propolis is a resinous [illegible] found in beehives. Collected by bees from buds [illegible] tion: Lewkovitsch, *Chemical Technology and [illegible] Oils, Fats and Waxes* 5th ed, vol. 2, p 896 (Ma[illegible]

ibid. 79, 6483 (1957); Schmidt, Grafen, *Ber.* 92, 1177 (1959); Reed, U.S. pats. 2,980,716 and 3,049,549 (1961, 1962 to Res. Corp.); Lewis, Raphael, *J. Chem. Soc.* 1962, 4263; Ose *et al.*, U.S. pat. 3,223,712 (1965 to Yamanouchi). *Reviews:* International Symposium on Thioctic Acid, Naples 1955; *C.A.* 51, 8153c (1957); Schmidt *et al.*, *Angew. Chem.* (Internatl Ed. in English) 4, 846–856 (1965), contains 190 refs.



dl-Form: Crystals from cyclohexane, mp 60–61°. bp 160–165°. U.v. absorption spectrum: Calvin, *Federation Proc.* 13, 703 (1954). Practically insol in water. Soluble in fat solvents. Forms a water-soluble sodium salt.

d-Form (the natural isomer): Crystals by vacuum sublimation (at 85–90° and 25 microns). mp 46–48° (microblock). $[\alpha]_D^{28}$ +104° (c = 0.88 in benzene). Absorption max: 333 mμ (ε = 150 in methanol). pKa 5.4. Practically insol in water, sol in fat solvents.

Sodium salt, $C_8H_{13}NaO_2S_2$, white powder, sol in water. pH of aq solns about 7.4.

l-Form: Crystals from cyclohexane, mp 45–47.5° (microblock). $[\alpha]_D^{28}$ −113° (c = 1.88 in benzene). Absorption max: 330 mμ (ε = 140 in methanol).

Thiocyanic Acid. Hydrogen thiocyanate; *German:* Rhodanwasserstoffsäure. HSCN; mol wt 59.09. C 20.33%, H 1.71%, N 23.71%, S 54.25%. Thiocyanic acid is believed to be a tautomeric mixture of HSCN and HNCS (*isothiocyanic acid*): Beard, Dailey, *J. Chem. Phys.* 18, 1437 (1950). Conveniently prepd in the laboratory according to the eq $KNCS + KHSO_4 \rightarrow K_2SO_4 + HNCS$: Birckenbach, Bucher, *Ber.* 73, 1153 (1940). Dilute aq solns may be prepd from ammonium thiocyanate by the action of ion exchange resins: Klement, *Z. Anorg. Allgem. Chem.* 260, 268 (1949).

Colorless gas or white solid depending upon degree of polymerization. Freely sol in water. Very strong acid. A 5% aq soln may be kept in the ice box for several weeks. Also sol in some organic solvents.

Human Toxicity: Inorganic thiocyanates may produce vomiting, excitation, delirium, convulsions. Chronic exposure may cause coryza, skin rashes, a variety of CNS symptoms. Cyanide effect does not occur.

2,2′-Thiodiethanol. Thiodiglycol; thiodiethylene glycol; bis(hydroxyethyl)sulfide. $HOCH_2CH_2SCH_2CH_2OH$; mol wt 122.18. $C_4H_{10}O_2S$; C 39.32%, H 8.25%, O 26.19%, S 26.24%. Prepd from ethylene oxide and hydrogen sulfide: Chichibabin, Bestuzher, *Compt. Rend.* 200, 242 (1935); Nenitzescu, Scarlatescu, *Ber.* 68, 587 (1935); Headlee, cited in G. O. Curme, F. Johnston, *Glycols* (Reinhold, New York, 1952), p 103.

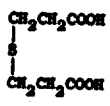
Liquid. d_4^{20} 1.1824. mp −16°. bp_{14} 168°. n_D^{20} 1.519. Miscible with water, alcohol. Slightly sol in ether.

Thiodiglycolic Acid Dimethylsulfide-α,α′-dicarboxylic acid; mercaptodiacetic acid. $HOOCCH_2SCH_2COOH$; mol wt 150.15. $C_4H_6O_4S$; C 31.99%, H 4.03%, O 42.62%, S 21.36%. Prepn from sodium chloroacetate and hydrogen sulfide: Loven, *Ber.* 27, 3059 (1894).

Crystals from water. mp 129°. Soluble in water or alcohol.

USE: Detection of copper, lead, mercury, silver: Dubsky, *et al.*, *C.A.* 34, 6185 (1940).

3,3′-Thiodipropionic Acid. β,β-Thiodipropionic acid; thiodihydracrylic acid; diethyl sulfide 2,2′-dicarboxylic acid. $C_6H_{10}O_4S$; mol wt 178.20. C 40.44%, H 5.66%, O 35.91%, S 17.99%. Prepn: Brit. pat. 571,628 (1945 to Am. Cyanamid Co.); Gresham, Shaver, U.S. pat. 2,449,992 (1948 to B. F. Goodrich).



Nacreous leaflets from hot water, mp 134°. K at 25° = 7.8×10^{-5}. One gram dissolves in 26.9 ml water at 26°. Freely sol in hot water, alcohol, acetone.

USE: Antioxidant for soap products and polymers of ethylene. In plasticizers and lubricants. Proposed for edible fats, oils, other foods.

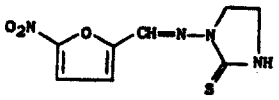
Thioformamide. $HCSNH_2$; mol wt 61.11. CH_3NS; C 19.65%, H 4.95%, N 22.92%, S 52.47%. Prepd by the action of phosphorus pentasulfide on formamide: Willstätter, Wirth, *Ber.* 42, 1911 (1909). Improved procedures: Erlenmeyer, Menzi, *Helv. Chim. Acta* 31, 2071 (1948). Schmitz, U.S. pat. 2,682,558 (1954 to Du Pont).

Prisms from ethyl acetate or from ether + petr ether. Turns yellow and dec to a dark-colored mass. May be stored as soln in abs ether, preferably over P_2O_5. mp 29°. Soluble in water (with considerable cooling). Freely sol in tetrahydrofuran, alcohol, ether, acetone, ethyl acetate; sparingly sol in cold chloroform, benzene, petr ether, carbon disulfide. The decompn products are hydrogen cyanide, hydrogen sulfide, ammonia, and some solid, amorphous, sulfur-contg compds.

Monohydrate, $CH_3NS.H_2O$, yellow oil.

USE: In the synthesis of the thiazole part of vitamin B_1.

Thiofurandene. *1-(5-Nitrofurfurylideneamino)-2-imidazolethione;* NF 441. $C_8H_5N_4O_3S$; mol wt 240.25. C 40.00%, H 3.35%, N 23.32%, O 19.98%, S 13.35%. Prepn: Michels, U.S. pat. 2,920,074 (1960 to Norwich Pharmacal Co.).



Crystals from nitromethane, dec above 225°. Solubility in water: 16 mg/l.

VET USE: Swine anthelmintic.

Thioglycerol. α-Monothioglycerol; thioglycerin. $HOCH_2CH(OH)CH_2SH$; mol wt 108.16. $C_3H_8O_2S$; C 33.31%, H 7.46%, O 29.59%, S 29.65%. Prepd by heating glycerol-α-monochlorohydrin with KHS in alcoholic soln. *Ref:* Sutton, *J. Am. Med. Assoc.* 104, 2168 (1935).

Yellowish, very viscous liq; slight sulfidic odor. d 1.295. Slightly sol in water; miscible with alcohol; insol in ether.

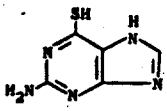
MED and VET USE: Has been used in 1:5000 concn to promote wound healing.

Thioglycolic Acid. Thioglycollic acid; *mercaptoacetic acid.* $HSCH_2COOH$; mol wt 92.12. C 26.07%, H 4.38%, O 34.74%, S 34.81%. Prepd by the action of sodium sulfhydrate on sodium chloroacetate; by electrolysis of dithioglycollic acid (from sodium sulfide and sodium chloroacetate).

Liquid. Strong, unpleasant odor. Readily oxidized by air. d 1.325. mp −16.5°. bp_{20} 123°. bp_{15} 108°. bp_5 96°. K_1 at 25° = 2.1×10^{-4}; $K_2 = 2.1 \times 10^{-11}$. Miscible with water, alcohol, ether, chloroform, benzene, and many other organic solvents. LD_{50} orally in rats: 0.15 ml/kg.

USE: Sensitive reagent for iron, molybdenum, silver, tin. With ferric iron a blue color appears, and when an alkali hydroxide is added to a soln contg ferrous salts and thioglycolic acid, a yellow precipitate forms. Used in the manuf of thioglycolates. The ammonium and sodium salts are commonly used for cold waving and the calcium salt is a depilatory. The sodium salt also is used in bacteriology in the prepn of thioglycolate media. *Human Toxicity:* Can cause severe burns and blistering of skin. Liberates hydrogen sulfide on decompn. *See* Hydrogen Sulfide.

Thioguanine. *2-Aminopurine-6-thiol.* $C_5H_5N_5S$; mol wt 167.21. C 35.92%, H 3.01%, N 41.89%, S 19.18%. Prepn: Elion, Hitchings, *J. Am. Chem. Soc.* 77, 1676 (1955); Hitchings, Elion, U.S. pats. 2,697,709, 2,800,473, 2,884,667 and Hitchings *et al.*, U.S. pats. 3,019,224 and 3,132,144 (1954, 1957, 1959, 1962, and 1964, all to Burroughs Welcome & Co.).



Needles from water, mp >360°.

MED USE: Exptl antineoplastic agent.

Japanese Journal of Cancer Research.
[GANN, Vol. 44; December 1953]

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PRODUCTION OF GASTRIC LESIONS IN THE RAT BY THE DIET CONTAINING FATTY ACIDS*

(With Plates XVII-XXII)

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The development of gastric lesions in rats receiving diets containing fatty acids, such as propionic, butyric and valeric acids, was demonstrated in this laboratory. The results are reported in this paper.

EXPERIMENTAL PROCEDURES

The fatty acids fed to rats were propionic, butyric, valeric, capronic, capric, lauric and palmitic acids. And the severe hyperplasia of the forestomach of rats developed only in the rats which were maintained on the diet containing low fatty acids, namely, propionic, butyric and valeric acids, while none of the rats receiving other fatty acids listed above showed such a gastric lesion.

Both sexes of albino rats of mixed strain weighing 50 g to 80 g were maintained on rice diet, to which the fatty acid was mixed evenly. Usually, the amount of the fatty acid added was 5-10 per cent of the rice. But, in the case of the butyric acid, the initial amount was 1 per cent of rice, and it was gradually raised up to 10 per cent by two week intervals after the beginning of the experiment. A small amount of cod liver oil was introduced along with the fatty acid on the assumption that the dissipation of fatty acid from the surface of rice grains may be somewhat hindered by so doing and that the diet may not become deficient in vitamin A. No attempt was made to estimate the actual amount of fatty acid ingested by the rats as the volatility of fatty acid rendered any such attempt practically impossible. In view of the deficient nature of the diet, green vegetables were given to the rats from time to time.

The toxicity of fatty acids is not great when given with food and essentially when the amount is five per cent or less animals seem to thrive and show substantial increase in body weight. The body weight of animals was measured every two weeks.

In the course of the experiment the rats were killed by exsanguination. The stomachs were removed at once and placed in a formalin fixative. After slight

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fixation, the stomachs were cut along the greater curvature, and examined for gross lesions. Then, those showing gross abnormalities were taken for routine histological examination, using hematoxylin-eosin stain.

Results

1. Butyric acid diet.

In this experiment, the amount of butyric acid added to rice varied. It was started with a small amount, 10 cc per 1 kg of rice, and was gradually raised up to 100 cc per 1 kg. Duration of each dose was about two weeks. In one group of rats which were killed on the 350 th day, the diet was supplemented with the powder of the dried beef liver, brewer's yeast and cod liver oil to avoid the deficient nurishment.

One hundred and twenty rats receiving the butyric acid diet were killed on the various experimental periods: 10 rats on the 50 th day, 5 on the 75 th day, 35 on the 100 th day, 15 on the 135 th day, 18 on the 150 th day, 10 on the 350 th day and 5 on the 500 th day, and several examples of these rats are shown in the plates. Twenty-two rats died on the day between these experimental periods. The stomachs of these experimental rats were examined for gross lesions and were fixed in formalin, were embedded in paraffin and cut and stained routinely. Figs. 1–10 show the lesions in the forestomach of rats kept on butyric acid diet for various durations.

The gastric lesions of the rats which were killed on the various periods of experiment are summerized in Table 1. As shown in the table, the majority of the rats which received butyric acid diet more than 50 days showed more or less definite gastric lesions, such as hyperkeratosis, hyperplasia and papillomatosis. And the lesions of papillomas with keratin cysts are often developed in the rats of

Table 1. Gastric Lesions in the Forestomachs of Rats kept on Butyric Acid Diet.

Experimental Days	Number of Rats	Gastric Lesions in the Forestomach		
		Hyperkeratosis or Hyperplasia only	Moderate degree of papillomatous or warty lesions	Extensive papillomatous growths with numerous keratin cysts
50	10	1	8	1
75	5	0	5	0
100	35	3	30	2
135	15	0	6	9
150	18	3	13	2
350*	10	0	1	9
500	5	0	0	5

* Butyric acid diet was supplemented with beef liver powder, brewer's yeast and cod liver oil.

more than 135 experimental days.

In the rats fed butyric acid diet for a long period, 350 and 500 days, marked keratin cysts were easily visible on the serosal view. In the case of rats which were killed on the 350 th day, the degree of the gastric lesions was similar to that of 500 day case. Then it seemed that sufficient diets of beef liver, brewer's yeast and cod liver oil did not favour the occurrence of the lesions but the responsible factors could not be analysed. These gastric lesions, however, did not change into malignant even when the administration was long in period and the condition was very pronounced. No remarkable change was found in the glandular stomach.

2. Propionic acid diet.

In this experiment, only 5 rats were maintained on rice, to which propionic acid was added. The amount of propionic acid added was 50 cc per 1 kg of rice throughout the experiment. One rat died early in the course of the experiment, and remaining 4 rats were killed on the 110 th day. The result is shown in Table 2. In 3 of 4 cases, umbilicate or warty lesions were evident in the forestomach, and the remaining one, however, showed no gastric change. These lesions to the naked eye appeared as isolated crater-like warts with ulceration on their tops. Figs. 11–13 show these lesions.

Table 2. Gastric Lesions in the Forestomachs of Rats kept on Propionic and Valeric Acid Diets.

Diet	Experimental Days	Number of Rats	Gastric Lesions in the Forestomach		
			Macroscopically Normal	Umbilicate or warty lesions	Extensive papillomatous growths
Propionic Acid	110	4	1	3	0
Valeric Acid	115	5	2	0	3
	150	4	0	0	4

3. Valeric acid diet.

10 rats were administered on rice, to which valeric acid was added. The amount of valeric acid added was 50 cc per 1 kg of rice throughout the experiment. One rat died early in the experiment. Five rats were killed on the 115 th day, and the remaining four were sacrificed on the 150 th day.

The results of the above experiment are shown in Table 2. In the table, 3 of 5 rats which were 115 day cases showed umbilicate lesions scattered in the forestomachs and 2 others revealed no lesion. All 4 rats of 150 day period, showed remarkable papillomatous growths. These lesions caused by valeric acid feeding, however, were not accompanied with keratin cyst which was usually seen in the

butyric acid case. Figs. 14-16 show these cases.

4. Other fatty acids diets.

Ten rats each were administered the rice diet, to which capronic, capric, lauric, or palmitic acids was added each in the proportion of 10 per cent. In all cases, the duration of the administration was 150 days. No remarkable change was detected either in the forestomach or glandular stomachs of rats kept on these fatty acid diets.

Pathological Notes

Grossly, many of the forestomachs were slightly enlarged, the wall were opaque, gray or white, and indurated, showing numerous irregular protuberances on the external surface. When the stomachs were cut along the greater curvature, hyperplasia of the epithelial lining and large papillomatous growth were grossly evident occupying a large portion of forestomach of all rats. In the early course of the experiment the discrete umbilicate lesions were seen. These lesions varied from small to large sizes. To the naked eye these lesions appear as numerous papillomata or warty excrescences. And the limiting ridge was prominent. The glandular stomach, however, was quite intact in all experimental groups.

Microscopically the squamous epithelium lining of the forestomach showed hyperkeratosis or hyperplasia which were designated as the diffuse lesions The retia were increased numerically, elongated and broadened, sometimes interlaced and anastomosing, giving the appearance of sessile papillary formation. The characteristic lesion was papillomatosis which were designated as the umbilicate or warty lesion frequently found in the forestomachs. Occasionally, spurs of hyperplastic epithelium extended downward from the papillomatous growths and penetrated the muscularis mucosa (Fig. 9). Some of these were found deeply placed in the submucosa heterotopically. When rats were administered with butyric acid for more than 135 days, these proliferations were covered with keratin in concentric whorls and in a flamelike fibrillary arrangement (Fig 10). These keratin cysts, large or small, were found penetrating the muscularis mucosa. Very often, these cysts were grossly visible on the outside surface of the stomach. And, it is noteworthy that the keratin cyst was prominent in the case of butyric acid only.

Generally speaking, these gastric lesions which developed in rats fed low fatty acids are similar in kind. But it is most severe in the butyric acid feeding. And the lesions in the case of propionic acid feeding seemed to be rather destructive than those due to butyric or valeric acid. And the valeric case is less marked than butyric case in the grade. In all foregoing gastric lesions, no evidence of true infiltrative malignant growth was detected in any part.

Discussion and Conclusions

Since the discovery of Fibiger, many workers have tried by various means to induce malignant tumors in the stomachs of rodents, but for the most part without succes. And reviews of the literature have been published by Klein and Palmer,[1] Sugiura,[2] Ivy,[3] Barrett,[4] and recently by Peacock et al.[5] Salmon and Copeland[6,7] appeared to be the first to have described that the marked gastric lesions were induced in the forestomach of rats which were kept on diet of large amount of tributyrin or butyic acid supplement. The author[8] indicated previously that the slight lesions in the forestomach of rats were produced when acetic acid was added to the diet. In this paper the effect of the administration of various fatty acids to rats was investigated. And the development of the lesions in the forestomach of rats was demonstrated in the case of administration of low fatty acids, i.e., propionic, butyric and valeric acids. And these results in the butyric acid are in accordance with reports of Salmon and Copeland. These lesions however are not entirely due to the vitamin A deficient condition.[9]

Microscopic examination of the gastric lesions revealed mainly extensive benign hyperplasia, hyperkeratosis of the squamous epithelium lining, consisting in the development of numerous papillomata and warty excrescences and occupying a large portion of the forestomach. These lesions are most severe in the case of butyric acid among the fatty acids investigated for comparable periods of time. The gastric lesions caused by propionic acid feeding was somewhat destructive in nature. And valeric acid produced less damage to the epithelial lining than butyric acid. Numerous keratin cysts were found only in the case of butyric acid diet. However, the glandular stomach of rats receiving these fatty acids remained quite intact notwithstanding the lining of forestomach was extensively damaged. No remarkable change was found as the result of administration of high fatty acids, such as capronic, capric, lauric and palmitic acids.

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要　旨

脂肪酸添加飼料による白鼠の前胃変化

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白米に5～10％の割合で脂肪酸を添加した飼料を白鼠に与えることによって，動物の前胃粘膜に乳嘴腫様変化が生成されることが確められた。実験に際し，脂肪酸としては，プロピオン酸，酪酸，吉草酸，カプロン酸，カプリン酸，ラウリン酸並びにパルミチン酸を選んだ。しかし，実験の結果，いわゆる低級脂肪酸（プロピオン酸，酪酸並びに吉草酸）飼与の場合にのみ動物の前胃に変化がみとめられ，他の高級脂肪酸の際には著変がみられなかった。

これらの前胃変化は，いづれの脂肪酸の場合でも大体同じ傾向を示し，扁平上皮細胞の著しい角化並びに肥厚増殖が先行し，さらに乳嘴腫様変化にまで発展する。酪酸飼与の場合に限り，特徴あるKeratin cystの出現がみられる。しかし500日に亘る長期の飼養にも拘らず，腫瘍化することはなかった。

Name	Function, Usage	Levels of Use
Dehydroacetate, Sodium Dehydroacetic Acid	Preservatives	
	Cut or peeled squash. Up to	0.0065%
Diethyl Pyrocarbonate	Fermentation inhibitor in still wines	
	Added before or during bottling, up to	0.02%
	When tested 5 days after bottling, up to	0.0%
Ethylenediamine	Component of bacteriostat in sugar cane being processed. Up to	0.0001%
Ethylene Oxide-Methyl Formate Mixture	Mold and yeast control in dried and glaceed fruits	
	Methylformate (as formic acid), up to	0.02%
	Ethylene oxide, up to	0.005%
Ethyl Formate	Yeast and mold inhibitor	
	Bulk and package fumigant in raisins and dried currants. As formic acid (free and combined), up to	0.025%
	Cashew nuts. Up to	0.0015%
	Dried fruits and nuts. As formic acid, up to	0.02%
Formaldehyde	Component of defoamer used in yeast and beet sugar production, as a preservative. In final product, up to	0.000001% (0.01 ppm)
Formaldehyde, para-	Polymerized formaldehyde, obtained by concentrating formaldehyde solution	
	Maple sirup, from use in tree tap holes. As formaldehyde, up to	0.0002%
Oxytetracycline	In edible tissue of chickens and turkeys when carcass is dipped in solution of oxytetracycline. Up to	0.0007%
Peroxide, Hydrogen	Preservative, bactericide for milk and cheese	
Polymixin B	Antibiotic	
	Used as bactericide in yeast culture for beer	
	Concentration reduced to 0.05 ppm in tank	0.0015%
Propionate, Calcium Propionate, Sodium Propionic Acid	Mold and rope inhibitors	
	Bread, rolls, etc.	0.125 - 0.15%
	Poultry stuffing	0.18%
	Chocolate products	0.24 - 0.30%
	Processed cheese, up to	0.30%
	Cake, cupcakes	0.0001 - 0.25%
	Artificially sweetened fruit jelly and preserves. Up to	0.1%
Sodium Chloride	Preservative	
	Pickling agent, preservative for meats, vegetables, butter	
	Prevention of browning in cut fruit	

Name	Function, Usage	Levels of Use
Butylated Hydroxytoluene (Di-tertiary-Butyl-para-Cresol) (2,6-di-tertiary-Butyl-4-methyl phenol)	Antioxidant	
	Chewing-gum base. Alone or with butylated hydroxyanisole and/or propyl gallate, up to	0.1%
	Potato flakes and sweet-potato flakes. Up to	0.005%
	Defoamer component in yeast and beet-sugar production. Up to	0.1%
	Potato granules. Total antioxidant, up to	0.001%
	Dry breakfast cereals. Total antioxidant, up to	0.005%
	Emulsion stabilizers for shortenings. Alone or with butylated hydroxyanisole, up to	0.02%
	Enriched rice. Up to	0.0033%
	Animal fats and shortenings containing animal fats. Up to	0.01%
Carbon Monoxide (Combustion product gas)	Removing and displacing oxygen	
	Carbon monoxide, up to 4.5% of gas	
Dilauryl Thiodipropionate	Antioxidant	
	General food use, per cent of fat or oil content, up to	0.02%
Erythorbate, Sodium (Sodium Isoascorbate) Erythorbic Acid (Isoascorbic Acid)	Antioxidants	
	Pickling brine. Per 100 gal, up to	7.5 oz
	Meat products. Per 100 lbs, up to	3/4 oz
	Brine, solution. Up to	10.0%
	Beverages	0.006 - 0.01%
	Baked goods	0.02 - 0.05%
	Cured cuts; cured comminuted product to accelerate color fixing in curing, per 100 lbs, Sodium erythorbate, up to	7/8 oz
	Erythorbic acid, up to	3/4 oz
	Pickle in which pork and beef products are cured or packed, per 100 gal	75 oz
Gas, Combustion product (from the controlled combustion in air of butane, propane, or natural gas)	Removing and displacing oxygen in the processing, storage, or packaging of citrus products, vegetable fats, vegetable oils, coffee, and wine	
Gum Guaiac	Antioxidant in edible fats or oils (Equivalent antioxidant activity - 0.01%)	
	Beverages	0.04 - 0.06%
	Rendered animal fat or a combination of such fat and vegetable fat. Up to	0.1%
Lecithin	Antioxidant	
	Prepared breakfast cereal	0.0025 - 0.005%
	Candy	0.06 - 0.25%
	Sweet chocolate, milk chocolate, etc.	
	Bread, rolls, buns, etc.	
	Oleomargarine. Up to	0.5%
Nordihydroguaiaretic Acid (Dihydroguaiaretic acid, normal)	Antioxidant	
	Prepared pie-crust mix	0.01%
	Candy (of fat)	0.01%
	Lard, butter, ice cream	
	Gassed cream (pressure-dispensed whipped cream)	
	General food use. Per cent of fat or oil content including essential (volatile) oil content, up to	0.02%
	Rendered animal fat or a combination of such fat and vegetable fat. Up to	0.01%

Name	Function, Usage	Levels of
Thiodipropionic Acid	Antioxidant	
	General food use. Per cent of fat or oil content including essential (volatile) oil content of food, up to	0.02%
Tocopherols (Vitamin E)	Antioxidant	
	Essential oils	0.02 - 0.1
	Rendered animal fats or a combination of such fat and vegetable fat in quantities up to	0.03%
2,4,5-Trihydroxybutyrophenone	Used as an antioxidant alone or in combination with other antioxidants	
	Total antioxidant, up to	0.02%

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PEROYLPIPERIDENE
(Piperine)

PROPANAL
(See Propionaldehyde)

PROPANOIC ACID
(See Propionic acid)

ISOPROPANOL
(See Isopropyl alcohol)

1-PROPANOL
(See Propyl alcohol)

2-PROPANOL
(See Isopropyl alcohol)

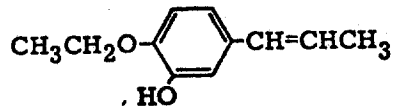
1-PROPENE-1,2,3-tri-CARBOXYLIC ACID
(See Aconitic acid)

PROPENE-1-THIOL
(Allyl mercaptan)

p-PROPENYLANISOLE
(See Anethole)

2-PROPENYL-6-ETHOXYPHENOL
(See Propenyl guaethol)

PROPENYLGUAETHOL
6-Ethoxy-m-anol

Chemical formula: 

Flavors in which used:
Chocolate, maple, nut, vanilla

Foods in which used:	Approx. Avg Maximum ppm
Beverages	5.9
Ice cream, ices	6.3
Candy	20
Baked goods	20
Puddings	2.5
Chocolate	25

4-PROPENYLGUAIACOL
(See Isoeugenol)

2-PROPENYL HEXANOATE
(See Allyl hexanoate)

4-PROPENYLVERATROLE
(See Isoeugenyl methyl ether)

PROPIONALDEHYDE

Chemical formula: CH_3CH_2CHO

Flavors in which used:
Fruit

Natural food occurrence:
Apples, onions

Foods in which used:	Approx. Avg Maximum ppm
Beverages	3.9
Ice cream, ices	12
Candy	11
Baked goods	13

PROPIONIC ACID

Chemical formula: CH_3CH_2COOH

Flavors in which used:
Butter, fruit

Natural food occurrence:
Apples, strawberries, tea, violet leaves (absolute)

Foods in which used:	Approx. Avg Maximum ppm
Beverages	1.2, 5.8
Ice cream, ices	6
Candy	14
Baked goods	20
Cheese	600

PROPYL ACETATE
Acetic acid, Propyl ester

Chemical formula: $CH_3COO(CH_2)_2CH_3$

Flavors in which used:
Berry, currant, raspberry, strawberry, apple, cherry, peach, pineapple, rum

Foods in which used:	Approx. Avg Maximum ppm
Beverages	4
Ice cream, ices	16
Candy	12
Baked goods	14

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972

TABLE 2 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP I) USED IN REGULAR FOODS(R)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
CALCIUM PHOSPHATE MONO (CONT.) NAS 0049	02	BREAK CERLS(R)	*	1.11000	1.11000
	03	OTHER GRAIN(R)	6	.24977	.52297
	10	MEAT PRODS(R)	*	.00300	.00400
	11	POULTRY(R)	*	.00300	.00400
	12	EGG PRODS(R)	*	.72000	.78000
	14	PROCSD VEGS(R)	*	.46061	.46061
	20	GELATIN PUD(R)	*	.26753	.26753
	22	SNACK FOODS(R)	*	.01300	.02200
	27	GRAVIES(R)	*	.20000	.20000
CALCIUM PHOSPHATE TRI NAS 0050 FEMA 3081	01	BAKED GOODS(R)	24	.06819	.14045
	02	BREAK CERLS(R)	5	1.25891	1.27363
	03	OTHER GRAIN(R)	9	.00249	.00377
	04	FATS OILS(R)	4	.04547	.07876
	05	MILK PRODS(R)	4	.76925	.76939
	07	FROZN DAIRY(R)	*	.00011	.00102
	09	FRUIT ICES(R)	*	.10000	.27000
	10	MEAT PRODS(R)	11	.01369	.02471
	11	POULTRY(R)	*	.13870	.16279
	12	EGG PRODS(R)	*	.18000	.18000
	13	FISH PRODS(R)	*	.04389	.05257
	15	CONDN RELSH(R)	*	.09528	.09528
	16	SOFT CANDY(R)	4	.02232	.07774
	19	SWEET SAUCE(R)	*	.49900	.50000
	20	GELATIN PUD(R)	8	.06000	.17969
	21	SOUPS(R)	4	.00255	.00390
	22	SNACK FOODS(R)	5	.08129	.18142
	23	BEV TYPE I(R)	15	.01445	.10359
	25	NUT PRODS(R)	*	.02500	.03000
	27	GRAVIES(R)	*	.01371	.04955
	28	IMIT DAIRY(R)	*	.29725	.31133
	31	CHEWING GUM(R)	*	********	********
	48	SEAS FLAVRS(R)	6	.10064	.13718
	49	MISC UNCLAS(R)	*	.05552	.20000
CALCIUM PHYTATE NAS 0051	15	CONDN RELSH(R)	*	.00200	.00200
CALCIUM PROPIONATE NAS 0052	01	BAKED GOODS(R)	32	.10611	.27966
CALCIUM SACCHARIN NAS 0054 FEMA 3528	01	BAKED GOODS(R)	*	.09756	.10000
	04	FATS OILS(R)	*	.03000	.03000
	07	FROZN DAIRY(R)	*	.03000	.03000
	08	PROCSD FRUT(R)	5	.01862	.02431

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TABLE 2 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP I) USED IN REGULAR FOODS(R)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
POTASSIUM PHOSPHATE DI (CONT.) NAS 0157	14	PROCSD VEGS(R)	*	.01108	.06377
	19	SWEET SAUCE(R)	*	.36500	.36500
	20	GELATIN PUD(R)	*	.15000	.20000
	23	BEV TYPE I(R)	*	.03180	.04648
	28	IMIT DAIRY(R)	9	1.39189	2.62647
POTASSIUM SORBATE NAS 0158 FEMA 2921	01	BAKED GOODS(R)	14	.06321	.07235
	04	FATS OILS(R)	12	.13611	.14445
	05	MILK PRODS(R)	4	.01975	.04869
	06	CHEESE(R)	8	.15158	.20347
	07	FROZN DAIRY(R)	6	.00505	.00989
	08	PROCSD FRUT(R)	9	.05837	.10181
	09	FRUIT ICES(R)	*	.06500	.07600
	10	MEAT PRODS(R)	*	.00020	.00030
	13	FISH PRODS(R)	*	.04280	.04280
	14	PROCSD VEGS(R)	*	.05052	.08419
	15	CONDM RELSH(R)	5	.03003	.05484
	16	SOFT CANDY(R)	10	.05421	.05786
	17	CONF FROST(R)	8	.08262	.08333
	18	JAM JELLY(R)	4	.05906	.09546
	19	SWEET SAUCE(R)	13	.05628	.06654
	20	GELATIN PUD(R)	8	.58049	.58380
	22	SNACK FOODS(R)	*	.03000	.08000
	23	BEV TYPE I(R)	12	.01635	.03556
	24	BEV TYPE II(R)	*	.02097	.02581
	27	GRAVIES(R)	*	.04894	.06413
	28	IMIT DAIRY(R)	*	.06500	.07500
	48	SEAS FLAVRS(R)	*	.01000	.01000
	49	MISC UNCLAS(R)	*	.10000	********
POTASSIUM SULFATE NAS 0159	23	BEV TYPE I(R)	*	.00987	.01481
DL-PROLINE FEMA 3377	10	MEAT PRODS(R)	*	.00200	.01000
	15	CONDM RELSH(R)	*	.00200	.01000
PROPIONIC ACID NAS 0163 FEMA 2924	01	BAKED GOODS(R)	17	.00750	.01203
	04	FATS OILS(R)	*	.00242	.00745
	06	CHEESE(R)	4	.00117	.00752
	07	FROZN DAIRY(R)	10	.00017	.00056
	16	SOFT CANDY(R)	10	.00033	.00099
	20	GELATIN PUD(R)	7	.00014	.00035
	22	SNACK FOODS(R)	*	.00002	.00004
	23	BEV TYPE I(R)	12	.00025	.00056

TABLE 2 — USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP I) USED IN REGULAR FOODS(R)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
PROPIONIC ACID (CONT.)	24	BEV TYPE II(R)	*	.00043	.00137
NAS 0163 FEMA 2924	29	IMIT DAIRY(R)	*	.00050	.00600
	30	HARD CANDY(R)	*	.00010	.00010
	31	CHEWING GUM(R)	*	.00020	.00020
PROPYLENE GLYCOL	01	BAKED GOODS(R)	44	.07903	.24409
NAS 0164 FEMA 2940	02	BREAK CERLS(R)	*	********	********
	03	OTHER GRAIN(R)	*	.00083	.00054
	04	FATS OILS(R)	14	.01429	.03179
	05	MILK PRODS(R)	*	.02299	.03771
	06	CHEESE(R)	7	.00711	.06199
	07	FROZN DAIRY(R)	30	.13749	.21258
	08	PROCSD FRUT(R)	5	.04941	.08202
	10	MEAT PRODS(R)	19	.02347	.05341
	11	POULTRY(R)	*	.00100	.01000
	12	EGG PRODS(R)	*	.00035	.00042
	14	PROCSD VEGS(R)	*	********	.50000
	16	SOFT CANDY(R)	40	.08945	.14408
	17	CONF FROST(R)	5	5.07337	5.08581
	19	SWEET SAUCE(R)	4	.30999	.42074
	20	GELATIN PUD(R)	17	.03609	.06854
	21	SOUPS(R)	4	.00003	.00004
	22	SNACK FOODS(R)	*	.00200	.09199
	23	BEV TYPE I(R)	35	.03775	.12390
	24	BEV TYPE II(R)	4	.00619	.58345
	25	NUT PRODS(R)	*	********	********
	26	RECONST VEG(R)	*	.00630	.00630
	27	GRAVIES(R)	4	.04901	.09803
	30	HARD CANDY(R)	23	.07150	.13531
	31	CHEWING GUM(R)	7	.06823	.29998
	48	SEAS FLAVRS(R)	*	14.90000	15.19895
	49	MISC UNCLAS(R)	*	.08098	.52750
PROPYL GALLATE	01	BAKED GOODS(R)	6	.00027	.00261
NAS 0165 FEMA 2947	02	BREAK CERLS(R)	*	********	********
	03	OTHER GRAIN(R)	*	.00010	.00010
	04	FATS OILS(R)	11	.00601	.01484
	07	FROZN DAIRY(R)	*	.00001	.00001
	10	MEAT PRODS(R)	4	.00299	.00993
	16	SOFT CANDY(R)	*	.00007	.00008
	17	CONF FROST(R)	*	.00010	.00010
	20	GELATIN PUD(R)	*	.00001	.00001
	21	SOUPS(R)	*	.00000	.00000
	22	SNACK FOODS(R)	5	.00100	.00296
	23	BEV TYPE I(R)	*	.00001	.00001

TABLE 2 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP I) USED IN REGULAR FOODS(R)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
SODIUM PHOSPHATE DI (CONT.)	27	GRAVIES(R)	8	.71421	.76866
NAS 0197 FEMA 2398	28	IMIT DAIRY(R)	8	.05531	.05695
SODIUM PHOSPHATE MONO	01	BAKED GOODS(R)	*	.80000	.90000
NAS 0198 FEMA 3534	02	BREAK CERLS(R)	*	.02000	.04610
	06	CHEESE(R)	*	.34174	.42162
	08	PROCSD FRUT(R)	*	.01500	.03000
	10	MEAT PRODS(R)	*	.00001	.00002
	11	POULTRY(R)	*	.49359	.98505
	12	EGG PRODS(R)	*	.50000	.50000
	14	PROCSD VEGS(R)	*	.02003	.07945
	16	SOFT CANDY(R)	*	.00185	.00349
	20	GELATIN PUD(R)	*	.16242	.17500
	22	SNACK FOODS(R)	*	.30000	.30000
	23	BEV TYPE I(R)	5	.04721	.11753
	28	IMIT DAIRY(R)	*	.30864	.30874
	31	CHEWING GUM(R)	*	.01750	.01750
SODIUM PHOSPHATE TRI	01	BAKED GOODS(R)	*	.10459	.18643
NAS 0199	02	BREAK CERLS(R)	*	.16600	.22800
	03	OTHER GRAIN(R)	*	********	********
	04	FATS OILS(R)	*	.46651	.46651
	06	CHEESE(R)	5	1.12355	2.67387
	10	MEAT PRODS(R)	*	.19686	.40000
	11	POULTRY(R)	*	.07068	.07077
	16	SOFT CANDY(R)	*	.00000	.00000
	20	GELATIN PUD(R)	*	.21400	********
	22	SNACK FOODS(R)	*	.37170	.38810
	23	BEV TYPE I(R)	*	.00290	.00290
	26	RECONST VEG(R)	*	********	********
	27	GRAVIES(R)	*	.10000	.20000
SODIUM PROPIONATE	01	BAKED GOODS(R)	20	.19717	.37023
NAS 0201 FEMA 3513	06	CHEESE(R)	4	.26918	.28903
	10	MEAT PRODS(R)	*	.00500	.01000
	16	SOFT CANDY(R)	*	.00911	.01258
	17	CONF FROST(R)	*	.00100	.00200
	20	GELATIN PUD(R)	*	.05000	.05000
	23	BEV TYPE I(R)	*	.01675	.02000
SODIUM PYROPHOSPHATE	01	BAKED GOODS(R)	4	.72253	1.36895
NAS 0202 FEMA 3625	05	MILK PRODS(R)	*	.00125	.00403
	06	CHEESE(R)	*	.09966	.12336

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FCCD CATEGORY AND TOTAL DIETARY, BASED ON FCCD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FCCD CATEGORY NO.	NAME	# OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH A	HIGH B
CALCIUM PHYTATE NAS 0051	15	CONDM RELSH(R)	*	0-5 MO.	**********	.002000	**********
				6-11 MO.	.016000	.044000	.016000
				12-23 MO.	.056000	.152000	.056000
				2-65+ YR.	.176000	.424000	.176000
CALCIUM PHYTATE NAS 0051	ALL CATEGORIES		*	0-5 MO.	**********	.002000	**********
	**********			6-11 MO.	.016000	.044000	.016000
	**********			12-23 MO.	.056000	.152000	.056000
	**********			2-65+ YR.	.176000	.424000	.176000
CALCIUM PROPIONATE NAS 0052	01	BAKED GOODS(R)	32	0-5 MO.	3.607740	4.774950	9.508440
				6-11 MO.	26.951940	54.964980	71.033640
				12-23 MO.	57.829950	95.286780	152.414700
				2-65+ YR.	145.582920	216.252180	383.693520
CALCIUM PROPIONATE NAS 0052	ALL CATEGORIES		32	0-5 MO.	3.607740	4.774950	9.508440
	**********			6-11 MO.	26.951940	54.964980	71.033640
	**********			12-23 MO.	57.829950	95.286780	152.414700
	**********			2-65+ YR.	145.582920	216.252180	383.693520
CALCIUM SACCHARIN NAS 0054	01	BAKED GOODS(R)	*	0-5 MO.	3.317040	4.390200	3.400000
				6-11 MO.	24.780240	50.536080	25.400000
				12-23 MO.	53.170200	87.608880	54.500000
				2-65+ YR.	133.852320	198.827280	137.200000
CALCIUM SACCHARIN NAS 0054	04	FATS OILS(R)	*	0-5 MO.	.150000	.150000	.150000
				6-11 MO.	.840000	2.250000	.840000
				12-23 MO.	1.890000	3.600000	1.890000
				2-65+ YR.	5.250000	9.480000	5.250000
CALCIUM SACCHARIN NAS 0054	07	FRCZN DAIRY(R)	*	0-5 MO.	.300000	1.230000	.300000
				6-11 MO.	2.850000	7.920000	2.850000
				12-23 MO.	4.320000	10.140000	4.320000
				2-65+ YR.	7.680000	18.510000	7.680000
CALCIUM SACCHARIN NAS 0054	08	PRCCSD FRUT(R)	5	0-5 MO.	.875140	2.346120	1.142570
				6-11 MO.	9.645160	24.019800	12.592580
				12-23 MO.	18.731720	37.184140	24.455860
				2-65+ YR.	22.027460	46.661720	28.758730
CALCIUM SACCHARIN NAS 0054	16	SOFT CANDY(R)	*	0-5 MO.	.020000	.200000	.040000
				6-11 MO.	.220000	.680000	.440000
				12-23 MO.	.350000	.930000	.700000
				2-65+ YR.	.580000	1.760000	1.160000

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	********** POSSIBLE DAILY INTAKE, MG. ********** (AGE)	AVERAGE	HIGH A	HIGH B
POTASSIUM SORBATE NAS 0158	ALL CATEGORIES ********** ********** **********	83	0-5 MO.	19.770760	32.322090	24.[illegible]
			6-11 MO.	165.624440	485.691400	226.[illegible]
			12-23 MO.	245.242860	548.282050	342.[illegible]
			2-65+ YR.	412.304020	887.837350	[illegible]
POTASSIUM SULFATE NAS 0159	23 BEV TYPE I(R)	*	0-5 MO.	.236880	.355320	.355440
			6-11 MO.	2.240490	7.668990	3.361870
			12-23 MO.	5.349540	16.038750	8.027020
			2-65+ YR.	10.264800	27.408950	15.402400
POTASSIUM SULFATE NAS 0159	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	.236880	.355320	.355440
			6-11 MO.	2.240490	7.668990	3.361870
			12-23 MO.	5.349540	16.038750	8.027020
			2-65+ YR.	10.264800	27.408950	15.402400
DL-PROLINE NAS 0161	10 MEAT PRODS(R)	*	0-5 MO.	.022000	.058000	.110000
			6-11 MO.	.414000	1.116000	2.070000
			12-23 MO.	.604000	1.033000	3.020000
			2-65+ YR.	1.560000	2.692000	7.[illegible]
DL-PROLINE NAS 0161	15 CONDM RELSH(R)	*	0-5 MO.	**********	.002000	**********
			6-11 MO.	.014000	.044000	[illegible]
			12-23 MO.	.056000	.152000	[illegible]
			2-65+ YR.	.176000	.424000	.880000
DL-PROLINE NAS 0161	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	.022000	.060000	.110000
			6-11 MO.	.430000	1.160000	2.150000
			12-23 MO.	.660000	1.190000	3.[illegible]
			2-65+ YR.	1.744000	3.020000	6.[illegible]
PROPIONIC ACID NAS 0163	01 BAKED GOODS(R)	17	0-5 MO.	.255000	.337500	[illegible]
			6-11 MO.	1.905000	3.[illegible]	3.[illegible]
			12-23 MO.	4.087500	6.735000	[illegible]
			2-65+ YR.	10.290000	15.285000	16.[illegible]
PROPIONIC ACID NAS 0163	04 FATS OILS(R)	*	0-5 MO.	.012100	.012100	.031250
			6-11 MO.	.067100	.281500	[illegible]
			12-23 MO.	.192400	.290100	[illegible]
			2-65+ YR.	.423500	.756000	[illegible]
PROPIONIC ACID NAS 0163	06 CHEESE(R)	4	0-5 MO.	**********	.001170	**********
			6-11 MO.	.031590	.113400	[illegible]
			12-23 MO.	.091260	.250740	[illegible]
			2-65+ YR.	.109580	.274100	[illegible]

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE -- SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	********** POSSIBLE DAILY INTAKE, MG. ********** (AGE)	AVERAGE	HIGH A	HIGH B
PROPIONIC ACID NAS 0163	07 FROZN DAIRY(R)	10	0-5 MO.	.001700	.003970	[illegible]
			6-11 MO.	.016150	.044800	[illegible]
			12-23 MO.	.024480	.057460	[illegible]
			2-65+ YR.	.043520	.104890	[illegible]
PROPIONIC ACID NAS 0163	16 SOFT CANDY(R)	10	0-5 MO.	.000440	.000600	[illegible]
			6-11 MO.	.007240	.022440	[illegible]
			12-23 MO.	.011550	.030000	[illegible]
			2-65+ YR.	.019140	.058030	[illegible]
PROPIONIC ACID NAS 0163	20 GELATIN PUD(R)	7	0-5 MO.	[illegible]	[illegible]	[illegible]
			6-11 MO.	[illegible]	[illegible]	[illegible]
			12-23 MO.	.019320	.047040	.045300
			2-65+ YR.	.028160	.073000	.071400
PROPIONIC ACID NAS 0163	22 SNACK FOODS(R)	*	0-5 MO.	**********	[illegible]	**********
			6-11 MO.	[illegible]	[illegible]	[illegible]
			12-23 MO.	[illegible]	[illegible]	[illegible]
			2-65+ YR.	.000260	.000740	[illegible]
PROPIONIC ACID NAS 0163	23 BEV TYPE I(R)	12	0-5 MO.	.006000	.009000	[illegible]
			6-11 MO.	.056750	[illegible]	[illegible]
			12-23 MO.	.135000	[illegible]	[illegible]
			2-65+ YR.	.260000	[illegible]	[illegible]
PROPIONIC ACID NAS 0163	24 BEV TYPE II(R)	*	0-5 MO.	.000000	.000000	[illegible]
			6-11 MO.	**********	.000400	**********
			12-23 MO.	**********	[illegible]	**********
			2-65+ YR.	.139750	[illegible]	[illegible]
PROPIONIC ACID NAS 0163	28 IMIT DAIRY(R)	*	0-5 MO.	.000000	.000000	[illegible]
			6-11 MO.	.007000	.011500	[illegible]
			12-23 MO.	.004000	.017000	[illegible]
			2-65+ YR.	.004500	.007500	[illegible]
PROPIONIC ACID NAS 0163	30 HARD CANDY(R)	*	0-5 MO.	.000000	[illegible]	[illegible]
			6-11 MO.	.000100	[illegible]	[illegible]
			12-23 MO.	.000300	.000900	.000300
			2-65+ YR.	.000600	.001700	[illegible]
PROPIONIC ACID NAS 0163	31 CHEWING GUM(R)	*	0-5 MO.	**********	**********	**********
			6-11 MO.	.000200	[illegible]	[illegible]
			12-23 MO.	.000200	.000600	[illegible]
			2-65+ YR.	.000400	.000800	.000400

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TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), FOR FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE --- SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
PROPIONIC ACID NAS 0163	ALL CATEGORIES ********** ********** **********	25	0-5 MO.	.278250	.377140	.474290
			6-11 MO.	2.109610	4.508530	3.790930
			12-23 MO.	4.526790	7.648560	[illegible]
			2-65+ YR.	11.320210	17.673220	19.871130
PROPYLENE GLYCOL NAS 0164	01 BAKED GOODS(R)	44	0-5 MO.	2.687020	3.550340	[illegible]
			6-11 MO.	20.073520	40.937940	[illegible]
			12-23 MO.	43.071350	70.965940	133.027650
			2-65+ YR.	108.429560	161.081140	[illegible]
PROPYLENE GLYCOL NAS 0164	02 BREAK CEBLS(R)	*	0-5 MO.	**********	**********	**********
			6-11 MO.	**********	**********	**********
			12-23 MO.	**********	**********	**********
			2-65+ YR.	**********	**********	**********
PROPYLENE GLYCOL NAS 0164	03 OTHER GRAIN(R)	*	0-5 MO.	.004150	.014110	[illegible]
			6-11 MO.	.050810	.237330	[illegible]
			12-23 MO.	.136120	.314070	[illegible]
			2-65+ YR.	.230740	.503020	.235920
PROPYLENE GLYCOL NAS 0164	04 FATS OILS(R)	14	0-5 MO.	.071450	.071450	[illegible]
			6-11 MO.	.400120	1.077770	[illegible]
			12-23 MO.	.900370	1.715000	[illegible]
			2-65+ YR.	2.900790	4.519540	[illegible]
PROPYLENE GLYCOL NAS 0164	05 MILK PRODS(R)	*	0-5 MO.	1.241460	.919460	[illegible]
			6-11 MO.	[illegible]	[illegible]	[illegible]
			12-23 MO.	[illegible]	[illegible]	[illegible]
			2-65+ YR.	[illegible]	[illegible]	[illegible]
PROPYLENE GLYCOL NAS 0164	06 CHEESE(R)	7	0-5 MO.	**********	.007110	**********
			6-11 MO.	.191970	.639670	[illegible]
			12-23 MO.	[illegible]	[illegible]	[illegible]
			2-65+ YR.	.668340	[illegible]	[illegible]
PROPYLENE GLYCOL NAS 0164	07 FROZN DAIRY(R)	30	0-5 MO.	[illegible]	[illegible]	[illegible]
			6-11 MO.	13.061550	[illegible]	[illegible]
			12-23 MO.	19.798560	[illegible]	[illegible]
			2-65+ YR.	[illegible]	[illegible]	[illegible]
PROPYLENE GLYCOL NAS 0164	08 PROCSD FRUT(R)	5	0-5 MO.	[illegible]	[illegible]	[illegible]
			6-11 MO.	[illegible]	[illegible]	[illegible]
			12-23 MO.	49.706460	[illegible]	[illegible]
			2-65+ YR.	58.452030	123.921460	97.025660

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
SODIUM PHOSPHATE TRI NAS 0199	27 GRAVIES(R)	*	0-5 MO.	.100000	.300000	.200000
			6-11 MO.	1.400000	3.900000	2.800000
			12-23 MO.	3.600000	10.200000	7.200000
			2-65+ YR.	8.300000	21.300000	16.600000
SODIUM PHOSPHATE TRI NAS 0199	ALL CATEGORIES ********** ********** **********	16	0-5 MO.	16.573070	28.473330	15.130620
			6-11 MO.	201.745630	551.309860	274.532720
			12-23 MO.	363.863680	769.179780	538.666060
			2-65+ YR.	696.970490	1309.511480	984.499620
SODIUM PROPIONATE NAS 0201	01 BAKED GOODS(R)	20	0-5 MO.	6.703780	8.872650	12.587820
			6-11 MO.	50.081180	102.134060	94.030420
			12-23 MO.	107.457650	177.055660	201.775350
			2-65+ YR.	270.517240	401.832460	507.955560
SODIUM PROPIONATE NAS 0201	06 CHEESE(R)	4	0-5 MO.	**********	.269180	**********
			6-11 MO.	7.267860	26.110460	7.803510
			12-23 MO.	20.998040	59.757960	22.544340
			2-65+ YR.	25.302920	63.526480	27.168820
SODIUM PROPIONATE NAS 0201	10 MEAT PRODS(R)	*	0-5 MO.	.055000	.145000	.110000
			6-11 MO.	1.035000	2.790000	2.070000
			12-23 MO.	1.510000	2.595000	3.020000
			2-65+ YR.	3.920000	6.505000	7.840000
SODIUM PROPIONATE NAS 0201	16 SOFT CANDY(R)	*	0-5 MO.	.018220	.182200	.025160
			6-11 MO.	.200420	.619480	.276760
			12-23 MO.	.318850	.847230	.440300
			2-65+ YR.	.528380	1.603360	.729640
SODIUM PROPIONATE NAS 0201	17 CONF FROST(R)	*	0-5 MO.	**********	.001000	**********
			6-11 MO.	.001000	.002000	.002000
			12-23 MO.	.002000	.007000	.004000
			2-65+ YR.	.003000	.008000	.006000
SODIUM PROPIONATE NAS 0201	20 GELATIN PUD(R)	*	0-5 MO.	1.000000	1.350000	1.000000
			6-11 MO.	6.400000	19.400000	6.400000
			12-23 MO.	6.900000	16.800000	6.900000
			2-65+ YR.	10.200000	26.250000	10.200000
SODIUM PROPIONATE NAS 0201	23 BEV TYPE I(R)	*	0-5 MO.	.402000	.603000	.400000
			6-11 MO.	3.802250	13.014750	4.540000
			12-23 MO.	9.078500	27.218750	10.840000
			2-65+ YR.	17.420000	46.514750	20.800000

TABLE 11, PART A -- ANNUAL POUNDAGE DATA FOR NAS APPENDIX A SUBSTANCES (GROUPS I & II)

SUBSTANCE NAME (SURVEY NO.)	# REPORTS TO NAS 1960/1970	POUNDAGE REPORTED TO NAS (MATCHING REPORTS FOR BOTH YEARS) 1960	1970	TOTAL 1970 POUNDAGE REPORTED TO NAS	# REPORTS TO FEMA	POUNDAGE REPORTED TO FEMA-- 1970 ONLY	TOTAL 1970 POUNDAGE NAS + FEMA
CALCIUM CITRATE NAS 0040	5/ 5	4,450	17,869	17,869			17,869
CALCIUM GLUCONATE NAS 0041	5/ 7	51,000	309,685	315,186			315,186
CALCIUM HYDROXIDE NAS 0044	17/ 19	411,033	844,306	868,172			868,172
CALCIUM LACTATE NAS 0045 FEMA 3535	6/ 9	51,550	27,037	73,337	*	233	73,570
CALCIUM OXIDE NAS 0046	4/ 4	2,000,000	10,230,040	10,230,040			10,230,040
CALCIUM PANTOTHENATE NAS 0047	14/ 14	950	6,247	7,996			7,996
CALCIUM PHOSPHATE DI NAS 0048 FEMA 3656	21/ 26	4,782,100	17,210,421	18,190,406	*	233	18,190,639
CALCIUM PHOSPHATE MONO NAS 0049	30/ 48	15,829,050	24,626,628	27,711,629			27,711,629
CALCIUM PHOSPHATE TRI NAS 0050 FEMA 3081	43/ 51	2,541,184	3,739,787	3,879,877	24	219,909	4,099,786
CALCIUM PHYTATE NAS 0051	*/ *	400	3,383	3,383			3,383
CALCIUM PROPIONATE NAS 0052	29/ 33	1,713,817	2,587,822	3,290,639			3,290,639
CALCIUM SACCHARIN NAS 0054 FEMA 3528	12/ 16	4,550	32,758	54,596	*	2,333	56,929
CALCIUM SILICATE NAS 0055	13/ 15	1,012,650	1,154,391	1,209,466			1,209,466
CALCIUM SULFATE NAS 0057	27/ 30	14,101,100	15,648,433	15,654,298			15,654,298
CAPRYLIC ACID FEMA 2799	/				20	1,534	1,534

TABLE 11, PART A --. ANNUAL POUNDAGE DATA FOR NAS APPENDIX A SUBSTANCES (GROUPS I & II)

SUBSTANCE NAME (SURVEY NO.)	# REPORTS TO NAS 1960/1970	POUNDAGE REPORTED TO NAS (MATCHING REPORTS FOR BOTH YEARS) 1960	1970	TOTAL 1970 POUNDAGE REPORTED TO NAS	# REPORTS TO FEMA	POUNDAGE REPORTED TO FEMA-- 1970 ONLY	TOTAL 1970 POUNDAGE NAS + FEMA
PROPIONIC ACID NAS 0163 FEMA 2924	*/ *	4,000	26,500	26,500	22	3,667	30,167
PROPYLENE GLYCOL NAS 0164 FEMA 2940	42/ 50	1,412,949	1,448,542	1,597,910	92	4,105,985	5,703,895
PROPYL GALLATE NAS 0165 FEMA 2947	13/ 15	80,901	96,420	97,020	7	40,126	137,146
PROPYLPARABEN NAS 0166 FEMA 2951	12/ 12	132	3,978	3,978	5	53	4,031
PYRIDOXINE HYDROCHLORIDE NAS 0167	19/ 20	2,400	16,834	17,314			17,314
RENNET NAS 0168	9/ 12	96,600	220,729	281,371			281,371
RIBOFLAVIN NAS 0169	39/ 45	73,800	113,970	121,105			121,105
RIBOFLAVIN 5-PHOSPHATE NAS 0170	*/ *	33	41	41			41
SACCHARIN NAS 0171 FEMA 3672	5/ 6	99	1,360	1,434	*	1,207	2,641
SILICA AEROGEL NAS 0174 FEMA 3506	14/ 16	66	23,277	29,627	*	6,666	36,293
SODIUM ACETATE NAS 0175 FEMA 3024	12/ 12	109,200	199,650	199,650	4	10,716	210,366
SODIUM ACID PYROPHOS NAS 0176	29/ 34	6,691,400	19,704,938	20,883,293			20,883,293
SODIUM ALGINATE NAS 0177 FEMA 2015	16/ 19	745,867	561,331	570,971	19	84,974	655,945
SODIUM ALUMINOSILICATE NAS 0178	17/ 20	3,305,600	4,352,536	4,591,182			4,591,182
SODIUM ALUMINUM PHOS NAS 0179 FEMA 3657	19/ 26	7,470,500	21,183,812	23,305,687	*	233	23,305,920

The free form of phenolphthalein was absent from the bile, but the small amount of material precludes the drawing of too dogmatic conclusions from this finding.

Summary.

The absence of the bile from the intestine reduced the absorption of free phenolphthalein, but had no influence on the amount of absorbed phenolphthalein glucuronide when conjugated phenolphthalein was excreted into the bile in the form of glucuronide only.

No free phenolphthalein was detected in the bile. Of administered free and conjugated phenolphthalein the free form was excreted more intensively.

Phenolphthalein glucuronide was excreted into the urine more easily than the free form.

The degree of absorption of free and conjugated phenolphthalein depended largely on the amount administered. The percentage absorption rose when the dosage was lowered.

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Pharmacological Effects of Fatty Acids, Triolein and Cottonseed Oil.

By

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Various triglycerides in emulsion show marked pharmacological properties on intravenous administration. For example, several with low molecular weights have been found distinctly toxic on intravenous injection (WRETLIND 1957 b; ORÖ, WESTERHOLM & WRETLIND 1959). On the other hand, fats of higher molecular weights, such as cottonseed oil, triolein and certain vegetable fats, show little or no toxicity on intravenous injection into mice. Hence it is possible to produce injectable emulsions of cottonseed oil as a carrier of agents, such as higher fatty acids, that owing to insolubility could not otherwise be studied for their pharmacological effects. Such fatty acid emulsions have enabled us to determine the toxicity of fatty acids on intravenous injection.

In the same investigation we have also studied the effects of these emulsions on respiration and blood pressure in cats. It was found that emulsions containing only cottonseed oil or triolein could cause reduced blood-pressure and apnoea. These effects were characterised by such pronounced tachyphylaxis that frequently they were noticeable only on the first injection. They were readily distinguishable, therefore, from the affects of the different fatty acid emulsions in which cottonseed oil acted as carrier.

Lastly, we conducted experiments on guinea pig small intestine in order to study the effects of fatty acid emulsions on smooth muscle.

Experimental.

Animal Experiments.

For *determinations of toxicity*, mice weighing 13–34 g were used. The emulsions or solutions of fatty acids were injected into the tail vein. Each fatty acid was administered to at least six groups each of 10 animals. The calculations of LD50 with their standard

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errors were by the method of MILLER & TAINTER (1944); the results are summarised in figure 1.

Some 100 cats weighing 2–6 kg were used for investigating the *respiratory* and *circulatory* responses to cottonseed oil, triolein and fatty acid emulsions. These animals were anaesthetized with diallymal (allobarbital (WHO), dial ®, Ciba, 25–40 mg per kg body weight) or chloralose (50 mg/kg). Respiration and blood pressure were recorded by means of a Grass electroencephalograph[1]) with supplementary demodulators. A Statham pressure transducer, model P 23 AA[2]), was used for blood-pressure determinations on the carotid artery. The respiration was recorded with a Grass pressure transducer PT-5 connected to a side tube in a tracheal cannula.

In a number of experiments the *pulmonary arterial pressure* was measured via a plastic catheter that had been sutured in the artery after opening the thorax. In one experiment the thorax was closed again; in the others it was left open under artificial respiration.

For studying the direct effect of fatty acids upon the heart, a *heart-lung preparation* described by WRETLIND (1957 b) was used. Blood pressure and flow were recorded by the Grass transducers mentioned above.

The action of fatty acids on guinea pig *small intestine* was studied in an isolated organ bath containing 15 ml Tyrode solution (0.8 %NaCl; 0.02% KCl; 0.02%$CaCl_2$; 0.02% $MgCl_2 \cdot 6\,H_2O$; 0.1% $NaHCO_3$; 0.005% $NaH_2PO_4 \cdot H_2O$; and 0.1% glucose). The organ bath was aerated with air. A Grass force-displacement transducer, model FT-03, was employed for recording the tension of the gut. In this connection we also studied the ability of fatty acids to abolish contractions elicited by histamine. For this purpose histamine (0.2 µg/15 ml) was first added to the organ bath and fatty acid emulsion was added without prior washing when maximal contraction had been obtained. The observed relaxation of the intestine was recorded.

Test Solutions and Test Emulsions.

For the *toxicity determinations* in mice the lower *fatty acids* (acetic, propionic, butyric, isovaleric, norvaleric, caproic and heptylic) were administered in aqueous solution, whereas the higher fatty acids, because of their insolubility in water, were emulsified. To prepare these *emulsions* the fatty acids were first dissolved in heated cottonseed oil, which served as carrier. Phosphatides, sodium cholate and glycerin monostearate were used as emulsifiers (table 1). The emulsions were homogenized in a Logeman apparatus (WRETLIND 1957 a). The pH of the solutions and emulsions was adjusted to 7.3 with NaOH.

The concentrations of fatty acids were so chosen that the injected volume required for determining LD50 amounted to between 3 and 45 ml per kg body weight. Thus acetic, propionic, butyric, isovaleric, norvaleric and caproic acids were administered as 10% and heptylic acid as 5% aqueous solutions; caprylic, nonanoic, capric, undecylic, lauric, tridecylic and myristic acids as 2% emulsions; pentadecylic, palmitic, margaric and stearic acids as 0.2%, and oleic acid as 2% emulsions.

For investigating the action of fatty acids on *guinea-pig gut*, 1% emulsions of the compositions recorded in table 1 were used.

Cottonseed oil was similarly utilized as carrier when investigating the *respiratory* and *blood-pressure* responses of the cat to higher fatty acids. Emulsification was with phosphatides and Pluronic F 68 (table 2), because they make stabler emulsions of

[1]) Grass Instrument Co., Quincy, Mass., U.S.A.
[2]) Statham Instruments Inc., 254 Carpenter Road, Hato Rey, Puerto Rico.

Table 1.

Composition of fatty acid emulsions used for determining the toxicity in mice.

The cottonseed oil, supplied by Wesson Oil and Snowdrift Sales Co., New Orleans, La., U.S.A., was used after filtration at +4°C. The phosphatides were prepared as described WRETLIND (1957 a). The emulsions were adjusted to pH 7.3 with N-NaOH.

Fatty acid	0.2–2 g
Cottonseed oil	10 g
Phosphatides	0.4 g
Sodium cholate	0.1 g
Glycerol monostearate	0.5 g
5% glucose solution to	100 ml

higher fatty acids than the emulsifiers previously mentioned. The fatty acids investigated were stearic, oleic, lauric and tridecylic.

The *cottonseed oil emulsions* used in the cat experiments had the compositons shown in table 2, except that they contained no fatty acid and that either unsaturated phosphatides (WRETLIND 1957 a) or hydrogenated phosphatides were used. In some experiments we studied emulsions containing triolein instead of cottonseed oil.

The *hydrogenated phosphatides* were prepared as described below. The phosphatides 320 g were dissolved in 600 ml cyclohexane: 3,400 ml absolute alcohol were added, and the precipitated cephalins were filtered off. The filtrate was concentrated under reduced pressure and nitrogen to a volume of 850 ml. To the resulting solution were added 30 mg Raney nickel as catalyst, the solution being then transferred to an autoclave in which it was subjected to hydrogenation at 85–130°C with hydrogen at a pressure of 75–150 atmospheres for two hours. The hydrogenated solution was filtered hot and passed through a column of 500 g Al_2O_3 with a diameter of 45 mm, equipped with a heating jacket (75°). The column was eluted with 95% ethanol. The first fraction of 300 ml was discarded; the next two litres of eluate were added to six litres off acetone and placed in a refrigerator at +4°. The resulting phosphatides were filtered of under reduced pressure, washed with acetone, and dried in a desiccator under reduced pressure. The yield amounted to 30–50 grams. Analysis showed N 1.6–1.7 per cent and P 3.7–3.8 per cent.

Table 2.

Composition of emulsions used for investigating the effects of fatty acids on respiration and blood pressure.

Pluronic F 68 (polypropylene-polyethylene-glucose) obtained from Wyandotte Chemical Corp., Wyandotte, Ill., U.S.A. The emulsions were adjusted to pH 7.3 with 1 N-NaOH.

Fatty acid	1 g
Cottonseed oil	10 g
Phosphatides	1.2 g
Pluronic	0.4 g
5% glucose solution to	100 ml

Results.

In attempts to determine the LD50 for the carrier emulsions with *cottonseed oil alone*, we found on intravenous injection of a maximal volume – i.e., 60 ml/kg of the 25% cottonseed oil emulsion with emulsifiers, as shown in table 1 – *into mice* only a transient depression of the respiration rate. It follows that the LD50 is higher than 15 g cottonseed oil per kilogram.

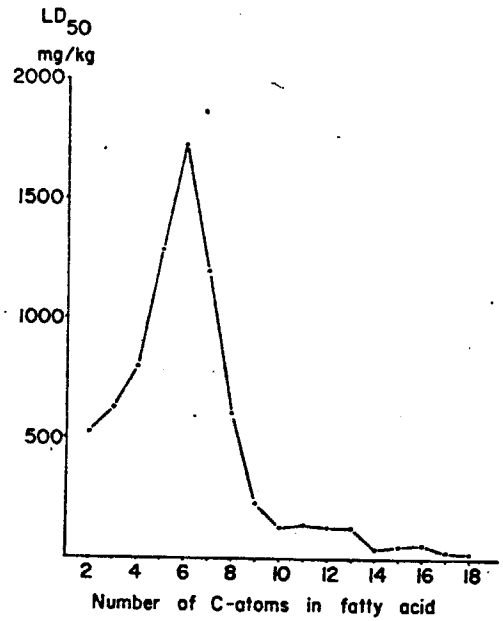

Fig. 1. *Toxicity of saturated fatty acids on intravenous injection into mice.* The abscissa indicates the number of carbon atoms in the fatty acid molecule; the ordinate, LD50 in milligrams per kilogram of body weight. The LD50 values in mg/kg, together with their standard errors, were: acetic acid 525 ± 21; propionic acid 625 ± 33; butyric acid 800 24; n-valeric acid 1290 ± 53; isovaleric acid 1120 ± 30; caproic acid 1725 ± 85; heptylic acid 1200 ± 56; caprylic acid 600 ± 24; nonanoic acid 224 ± 4.6; capric acid 129 ± 5.4; undecylic acid 140 ± 4.2; lauric acid 131 ± 5.7; tridecylic acid 130 ± 7.0; myristic acid 43 ± 2.6; pentadecylic acid 54 ± 3.2; palmitic acid 57 ± 3.4; margaric acid 36 ± 0.3; stearic acid 23 ± 1.3.

Figure 1 shows that the intravenous *toxicities of different fatty acids* differ considerably for mice. Least toxic is caproic acid with six carbon atoms, which has an LD50 of 1,725 ± 85 mg per kg body weight. With an increased or decreased number of carbon atoms in the fatty acid molecule, the toxicity rises. The most toxic of all those investigated was

stearic acid, which had an LD50 of 23 ± 0.7 mg per kg. Approximately the same value was obtained for stearic acid in the rat (21.5 ± 1.8 mg/kg). Oleic acid showed an LD50 of 230 ± 18 mg/kg in mice. This implies that stearic acid is ten times more toxic than the unsaturated oleic acid. The toxicity of norvaleric and isovaleric acids differed slightly, LD50 being 1,290 ± 53 and 1,120 ± 30 mg/kg, respectively.

When the fatty acid solutions and emulsions were injected in amounts near the LD50 dose, the animals immediately had convulsions and collapsed on their sides. Respiration ceased within 1–2 minutes, sometimes after hyperpnoea.

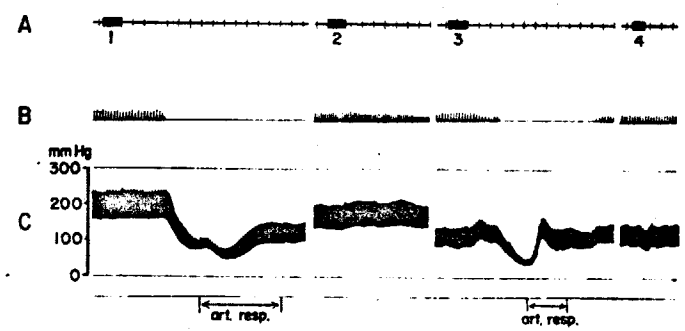

Fig. 2. *Influence of cottonseed oil emulsion on blood pressure and respiration.* Cat, 3.0 kg, under Dial ® anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. "Art. resp." denotes artificial respiration. At 1, 2, 3 and 4, 0.7 ml/kg of 10% cottonseed oil emulsion containing phosphatides was injected intravenously. The interval between 1 and 2 was four minutes, between 2 and 3 four hours and between 3 and 4 two minutes.

Intravenous administration of emulsion with *cottonseed oil or triolein alone to anaesthetized cats* produced various effects, depending on the dose and the emulsifiers. With soy-bean phosphatides and Pluronic F 68 as emulsifiers an initial intravenous injection (0.2–1 ml/kg body weight) sometimes caused a fall of blood pressure as well as apnoea (fig. 2). The latency was usually around 10–40 seconds. Another injection of the same dose invariably had either a less effect or none at all. – The action of these cottonseed oil emulsions is thus characterized by pronounced tachyphylaxis; hence the same or progressively higher doses can be repeated with no demonstrable effects on blood pressure and respiration. – When Pluronic F 68 alone was the emulsifier, 1–3 ml/kg was required to produce an effect with 10% cottonseed oil or triolein emulsion.

Cottonseed oil emulsions produced a rise of pressure in the *pulmonary artery* up to 100 mm Hg (fig. 3). The latency varied from 10 seconds to

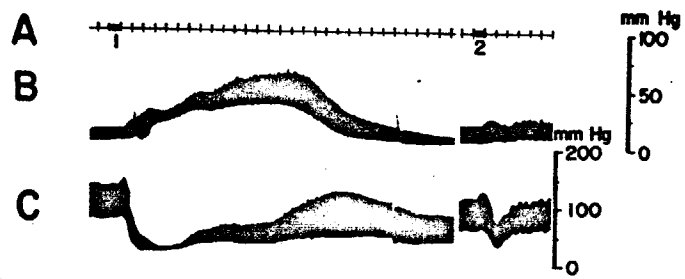

Fig. 3. *Effect of cottonseed oil emulsion on pressure in the pulmonary and carotid arteries.* Cat 3.3 kg, under chloralose anaesthesia with open thorax. A indicates 10-second intervals and injection times; B, the pulmonary artery; C, the carotid artery pressure. At 1 and 2, 0.5 ml/kg of a 10% cottonseed oil emulsion with phosphatides (1.2%) and pluronic (0.2%) as emulsifiers were injected.

2 minutes. Here too the effect declined with repeated injections and ultimately disappeared, although the injected doses were high.

The hypotensive action of these emulsions was substantially less after vagotomy, but in spite of this operation there was an unchanged rise of pressure in the pulmonary artery. This elevation in pressure showed a high degree of tachyphylaxis (fig. 5).

Direct experiments showed also that soyabean phosphatides caused a blood-pressure fall and apnoea, both of which effects exhibited marked tachyphylaxis. With repeated injections, however, the hypotensive action did not completely disappear (fig. 4), a result suggesting that two hypotensive factors were present here.

With cottonseed emulsions containing fatty acid, the first injections were sometimes accompanied by interference with the specific effects of

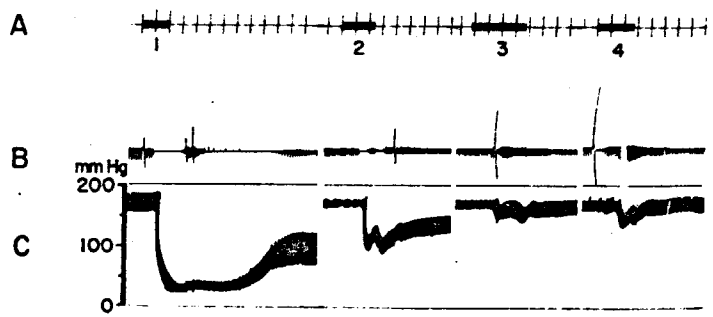

Fig. 4. *Effect of phosphatides on blood pressure and respiration.* Cat, 3.8 kg, under Dial ® anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, the blood pressure in mm Hg. At 1, 2, 3 and 4 4 ml/kg of 2.4% solution of soya bean phosphatides and 0.2% Pluronic F 68 were injected. The phosphatides have a hypotensive action characterized by tachyphylaxis, though a slight effect persists.

the cottonseed oil, but on repeated injection these quickly subsided because of the tachyphylaxis. With fatty acid emulsions containing hydrogenated phosphatides, only the effects of the fatty acids were observed.

Intravenous injection of the *fatty acids* investigated – stearic, oleic, lauric and tridecylic – had a hypotensive effect on the systemic *circulation*, but raised the pulmonary arterial pressure. The doses required for this action were 5 mg stearic acid, 50 mg oleic acid, 75 mg lauric acid or 60 mg tridecylic acid per kg body weight. All of the fatty acids studied had a negatively inotropic effect on the heart, as shown by the experiments on heart-lung preparations. Similar results were given by oleic acid emulsions

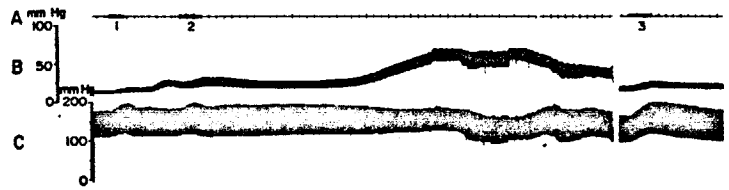

Fig. 5. *Influence of triolein emulsion on blood pressure and pulmonary arterial pressure after vagotomy.* Cat, 4.0 kg, under Dial ® anaesthesia with bilateral vagotomy. A indicates 10-second intervals and injection times; B, the pressure in the pulmonary artery; C, the arterial blood pressure in mm Hg. Four ml/kg of 10% triolein emulsion with pluronic F 68 only as emulsifier were injected intravenously at 1, and 7 ml/kg at 2 and 3. It will be seen that the emulsion caused a rise of blood pressure in the pulmonary artery with a slight secondary fall in blood pressure, effects that disappeared on repeated injection.

without cottonseed oil as carrier and also by those with unsaturated phosphatides and Pluronic F 68 as emulsifiers. Emulsions with different emulsifiers differed somewhat in their effects. For instance, the effect on pulmonary arterial pressure was far less with hydrogenated than with unsaturated phosphatides. The action on the *respiration* was characterized by hyperpnoea after small doses and apnoea after larger ones. No tachyphylaxis was observed with fatty acid emulsions. In several experiments, indeed, an increased effect was noted on repeated injection of equal doses (figures 6 and 7). – Vagotomy had no effect on falls in blood-pressure produced by the fatty acids studied.

As to the action of *fatty acid emulsions on small intestine from the guinea pig*, our results indicated that the emulsions did not themselves cause any contraction, even in relatively large doses (100 mg/15 ml). They could, however, inhibit the contraction produced by histamine. The degree of inhibition depended on the particular fatty acid contained in the emulsion. Fig. 8 shows the relative potencies of the fatty acids in this respect. It will be seen from the curve that capric and undecylic acids, i.e., those with 10 or 11 carbon atoms, were the most effective. Somewhat more than 15 mg/15 ml of these acids was required for total inhibition of

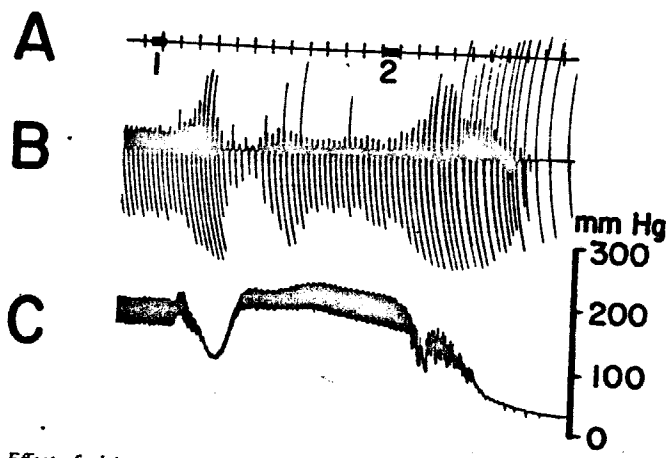

Fig. 6. *Effect of oleic acid emulsion on blood pressure and respiration.* Cat, 4.0 kg, under chloralose anaesthesia with bilateral vagotomy. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. One ml/kg of 10% oleic acid emulsion in cottonseed oil (10%) with soyabean phosphatides (1.2%) and pluronic F 68 (0.2%) as emulsifiers was injected at 1 and 2.

the contraction after 0.2 µg histamine. If the fatty acid molecule contained a greater or smaller number of carbon atoms than 10 or 11, the relaxing action on histamine-contracted intestine decreased; for instance, 150 mg of butyric acid was required for total inhibition. Comparison of undecylic acid with its unsaturated homologue, undecylenic acid, revealed no significant difference. The emulsion system alone without fatty acid is inert. No difference was detectable when the lower fatty acids were administered in aqueous solutions instead of in emulsions.

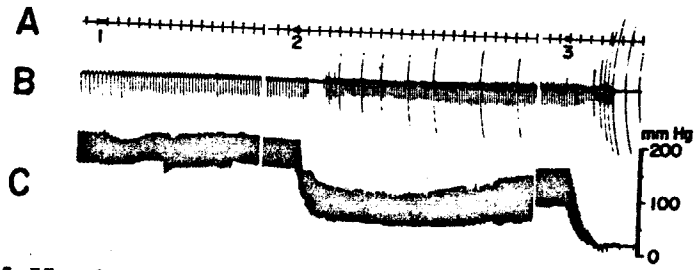

Fig. 7. *Effect of stearic acid emulsion on respiration and blood pressure.* Cat, 4.3 kg, under chloralose anaesthesia. A indicates 10-second intervals and injection times: B, respiration: C, blood pressure in mm Hg. At 1, 2 and 3, a 1% stearic acid emulsion with 10% cottonseed oil as carrier and phosphatides (1.2%) and pluronic F 68 (0.2%) as emulsifiers was injected at a dose of 0.2 ml/kg.

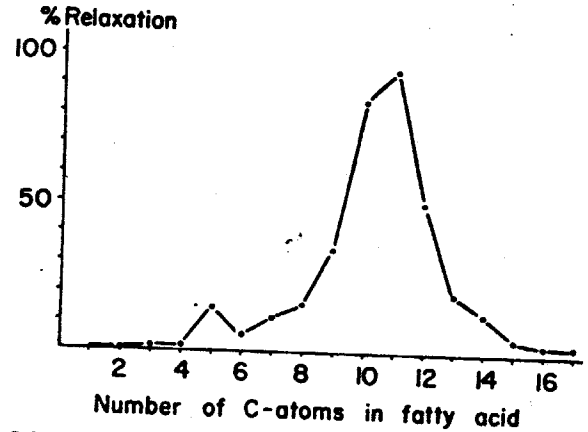

Fig. 8. *Relaxing action of fatty acids on guinea-pig small intestine contracted by histamine.* The fatty acids were administered in the form of emulsion. Abscissa indicates the number of carbon atoms in the fatty acid molecule. Ordinate shows percentage relaxation of guinea-pig intestine contracted by 0.2 µg histamine, after addition of 1.5 mg fatty acid to an organ bath containing 15 ml.

Discussion.

This investigation thus shows that several fatty acids are markedly toxic when administered intravenously. It is mainly the higher fatty acids that show this, (LD50 23 mg/kg) to the greatest extent. With a reduced number of carbon atoms in the molecule the toxicity diminishes, being least for caproic acid with six carbon atoms (LD50 1,724 mg/kg). When compared with the triglycerides of low molecular weight, the free fatty acids thus show the reverse behavior in toxicity; for the LD50 curve for triglycerides reveals a distinct minimum when the fatty acid component has five or six carbon atoms. Thus, hydrolysed fatty acids cannot be responsible for the acute toxic action of the triglycerides.

Our value for the LD50 of acetic acid (525 mg/kg) is closely consistent with that found by WELCH *et al.* (1944) – 380 mg sodium acetate per kg in mice. SAMSON, DAHL & DAHL (1956) described a hypnotic action of fatty acids of low molecular weight (C_2–C_{10}). There seems to be no direct correlation between hypnotic and toxic effects. The ED50 for hypnotic action decreases progressively with an increasing number of carbon atoms in the fatty acid molecule, in contrast to the LD50, the highest dose being that of caproic acid with six carbon atoms and the dose then falling with both increased and reduced numbers of carbon atoms.

The responses of respiration and blood pressure to the fatty acid emulsions differed from those to the triglycerides of low molecular weight

(WRETLIND 1957 a); in cats intravenous injection of more than 5 mg stearic acid or 50 mg oleic acid per kg was followed by apnoea and a fall in blood pressure as well as by convulsions with lethal outcome. Lower doses had a depressor action on blood pressure; the respiration sometimes being stimulated. The dose required for toxic action varied somewhat with the emulsifier employed. The effect on the circulatory system also differed with different emulsifiers. Stearic acid emulsions with phosphatides and Pluronic as emulsifiers raised the pulmonary arterial pressure substantially, and this elevation contributed to the fall of blood pressure in the systemic circulation. With hydrogenated phosphatides there was no demonstrable influence on pulmonary arterial pressure; on the other hand these emulsions had a pronounced effect on heart-lung preparations, so that their hypotensive action may be attributable to direct cardiac depression. Oleic acid emulsions that had been emulsified solely with phosphatides and Pluronic F68, without cottonseed oil, also had a hypotensive and toxic action on heart-lung preparations.

It is accordingly evident that the emulsified higher fatty acids studied had, on intravenous injection, a pronounced toxic action associated with a fall of blood pressure.

PELTIER (1956) determined the toxicity of some neutral fats and free fatty acids in non-emulsified form. He concluded that the toxic effects of fatty acid were caused by damage to the pulmonary capillaries, with extravasation into the alveolar spaces. JEFFERSON (1948) held a similar view. In our experiments on anaesthetized cats we occasionally observed progressive respiratory distress, and blood-tinged froth escaped from the tracheal cannula. At autopsy the lungs were oedematous. – It is worthy of note that the LD50 is more or less the same for non-emulsified oleic acid in rabbits (PELTIER 1956) and emulsified oleic acid in mice (250 mg/kg and 230/kg respectively). SCUDERI (1941) found that 0.33 ml of non-emulsified oleic acid per kg had a toxic action on the dog. The emulsified form had a lethal effect on anaesthetized cats in somewhat smaller doses, i.e., 50–100 mg/kg. However, total amounts substantially greater than 250 mg of emulsified oleic acid per kg can be given by repeatedly injecting sublethal doses.

The fatty acid emulsions had no stimulating action on *small intestine from the guinea pig* – a finding that accorded with results reported by GABR (1956). The emulsions could, however, relax histamine-contracted intestine. This effect was not correlated with their toxic action; rather the reverse, the relaxing effect being maximal for fatty acids with 10 or 11 carbon atoms and decreasing with either a smaller or greater number of carbon atoms in the molecule. Of interest in this connection is the finding of GANLEY *et al.* (1960) that higher fatty acids partially inhibited the action of serotonin (5-hydroxytryptamine) and the effect in anaphylactic shock in mice.

As to the blood pressure fall that follows injection of emulsions of *cottonseed oil* or *triolein*, it is difficult to determine the mechanism exactly because of the pronounced tachyphylaxis.

If an anaesthetized cat is injected with 6 ml/kg 10% emulsion of cottonseed oil or triolein emulsified with 0.1 or 1.0% Pluronic F 68, a substantial fall in blood pressure as well as apnoea occur after about 10–60 seconds. The cat may even die after this first injection. The pulmonary arterial pressure sometimes rises as high as 100 mm Hg. Our results suggest that the hypotensive effect is elicited mainly via the vagus, for it is practically eliminated by vagotomy (fig. 5).

Since the responses of blood pressure can also be obtained with the soya bean phosphatides alone, it seems probable that the effects observed with the cottonseed oil and triolein emulsions were due to the presence of such phosphatides. That the unsaturated phosphatides were responsible also appears likely, inasmuch as there was no circulatory response when hydrogenated soya bean phosphatides were used as emulsifiers. The fact that cottonseed oil emulsions with Pluronic F 68 as the sole emulsifier produced similar effects on the blood pressure shows that this is not the only explanation. Our observations tend rather to suggest that the effects of intravenously administered fat emulsions are due to a number of factors, such as the properties of the fat, the emulsifiers, the surface tension, size of fat particles and the charge carried by them. On the basis of our present knowledge in this field, it is impossible to say finally which is the principal factor involved.

According to SEEVERS (1958), tachyphylaxis is probably due to specific "receptors" that may take up the active substance, thereby eliciting the pharmacological action. When no more of these receptors are available, the effect ceases. The active substance is subsequently released slowly, and not until the liberation is complete can an effect be once more obtained. In our experiments the recovery period was relatively long, at least four hours. It is not yet possible, however, to say where these receptors are situated or by what mechanism the fat emulsions block them.

Summary.

Pharmacological investigations of the effects of intravenously administered fatty acids of high molecular weight are handicapped by the fact that such acids are insoluble in water at neutral reaction. By utilizing fat particles in an emulsion as carriers of these fatty acids, it has been possible

in our investigation to study the toxicity and certain pharmacological effects of such acids when given intravenously. Although emulsions of the carrier fat are devoid of toxicity, they exhibit peculiar pharmacological effects characterized by pronounced tachyphylaxis. In this investigation the following observations were made.

The LD50 by intravenous injection into mice was determined for saturated *fatty acids* from C_2 to C_{18}. Of these, stearic acid emulsion showed the highest toxicity: LD50 23 mg/kg body weight. With a decreasing number of carbon atoms in the fatty acid molecule the toxicity fell, reaching a minimum for caproic acid: LD50 1,725 mg/kg. As the number of carbon atoms declined beyond that point, so did the toxicity rise again, the LD50 amounting to 525 mg/kg for acetic acid.

The LD50 for stearic acid was ten times less than for its unsaturated analogue, oleic acid. For the *cottonseed oil emulsion* employed as "carrier" the LD50 was indeterminable, being higher than 15 g cottonseed oil per kilogram.

The pharmacological effects of emulsions of *cottonseed oil and triolein* on respiration and blood pressure were investigated by intravenous injection into cats. With soya bean phosphatides and Pluronic F 68 as emulsifiers a blood pressure fall and apnoea as well as an elevation of the pulmonary arterial pressure were observed. These effects were also noted for fat emulsions emulsified solely with Pluronic F 68 and for unsaturated soya bean phosphatides alone. Emulsions with hydrogenated phosphatides, on the contrary, did not produce such effects. The action on the circulation and respiration showed marked tachyphylaxis.

The effects of higher *fatty acid emulsions on circulation and respiration* were characterized by a fall in blood pressure as well as hyperpnoea or apnoea. Such emulsions also had a negatively inotropic effect on the heart.

Fatty acid emulsions had no direct action on *small intestine* from the guinea pig, but inhibited contractions elicited by histamine.

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THE EFFECT OF VOLATILE FATTY ACIDS ON PLASMA GLUCOSE CONCENTRATION*

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(*Received* 7 *January* 1966)

Abstract—1. Butyrate causes a marked increase in plasma glucose in lambs and a remission of hypoglycemic convulsions.

2. Propionate causes a similar remission but a considerably smaller plasma glucose increase.

3. Neither of these compounds materially affects plasma glucose levels in other species.

4. Acetate and β-hydroxybutyrate had no effect on plasma glucose concentration in any species.

5. Lamb liver perfusion results were variable as regards a direct action of butyrate on liver glycogen.

INTRODUCTION

VERY little ingested carbohydrate is absorbed as such in ruminants (Schambye, 1951) since the rumen microflora readily attack these materials and metabolize them to smaller compounds. The volatile fatty acids, acetic, propionic, and butyric acids, are considered to be major products of this rumen fermentation and to be a major energy source in ruminant metabolism (Hungate *et al.*, 1961). In spite of the lack of alimentary carbohydrate, the ruminants have a plasma glucose concentration (60–80 mg per cent) only slightly below other mammals and a relatively high rate of glucose utilization (Annison & White, 1961; Jarrett, Jones & Potter, 1964). This is accomplished by an active process of gluconeogenesis from absorbed nutrients, of which only propionate, among the volatile fatty acids, has been shown to provide a significant net source of glucose (Pennington, 1952, 1954; Annison *et al.*, 1963; Black *et al.*, 1961; Leng & Annison, 1963). Several workers have shown that intravenous infusion of proprionate and butyrate may increase the plasma glucose concentration (Reid, 1951; Potter, 1952; Kronfeld, 1957; Ash *et al.*, 1964). The exact mechanism by which butyrate causes a glucose increase remains

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obscure, but it is due, at least in part, to an activation of liver phosphorylase and subsequent breakdown of glycogen (Phillips *et al.*, 1965).

MATERIALS AND METHODS

Experimental animals were mixed whiteface lambs and Holstein calves from 5 to 90 days old. The dogs and the horse were mature animals of mixed breeding. All animals had sterile polyethylene catheters inserted into the jugular vein to allow frequent blood sampling with minimum disturbance for the animals. Catheter ends were fitted with a Tuohy–Borst adapter, a two-way stopcock, and the entire assemblage was anchored with a simple subcutaneous suture. The patency of the catheter was maintained by injection of heparinized (12 units/cm^3) sterile saline into the catheter after removing each blood sample. The volatile fatty acids and β-hydroxybutyrate, a ketone body, were administered intravenously as a 2·5 N solution, neutralized to pH 7·2 with NaOH; 2·5 mmoles/kg body wt. were given at each injection. Blood samples were taken at frequent intervals after the injection of the volatile fatty acids, immediately placed in heparinized, fluorinized tubes, chilled and centrifuged. The plasma was removed and frozen for subsequent analysis. Plasma glucose concentration was measured by a glucose oxidase method (Campbell & Kronfeld, 1961) after precipitation of the proteins with stable tungstic acid (Hycel Corporation). Ketone body concentration was measured in the distillate from the plasma protein-free filtrate using a method described by Reid (1960). Liver phosphorylase and glycogen were assayed by the methods of Illingworth & Cori (1953) and Hansen *et al.* (1952), respectively. Insulin (Regular Illetin, Ely Lilly Co.) was given at the rate of 4 units/kg/hr until convulsions appeared at which time one of the volatile fatty acids or β-hydroxybutyric was administered through the jugular catheter. The liver perfusion apparatus used to study *in vitro* responses to metabolites has been described in detail elsewhere (Phillips, 1965).

RESULTS

In the initial experiments the animals were made hypoglycemic with insulin before administration of the volatile fatty acids. This was done in part to confirm earlier work (Potter, 1952) and in part to test the hypothesis that butyrate is hyperglycemic only in conditions of hypoglycemia (Kronfeld, 1956). The results with lamb 73, shown in Fig. 1, essentially duplicate Potter's experiment in which butyrate was injected intravenously followed by propionate and a second butyrate injection. It can be seen that the injection of butyrate caused an increase in plasma glucose concentration, and that this effect was greater with butyrate than it was with an equimolar amount of propionate. An even greater response was obtained in a second lamb (No. 27), and again the effect of butyrate exceeded that of propionate. In the third lamb (No. 70), the sequence was reversed, injecting first propionate followed by butyrate and, finally, acetate. The response to the first propionate injection was relatively great, about a twofold increase in plasma glucose, but the subsequent response to butyrate was at least as great, if not greater.

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There was very little effect from the second injection of propionate and no detectable response to acetate injection.

To test the durability of the response to butyrate, two lambs were injected repeatedly for a period of more than 3 hr. The results, summarized in Fig. 2, show that the response diminished with time, the plasma glucose increase being only one-fifth as great after the fourth successive butyrate injection as it was after the first injection (lamb 46). In lamb 47 (Fig. 2) the plasma ketone body concentration was also measured and found to increase three- to fivefold immediately after each injection of butyrate.

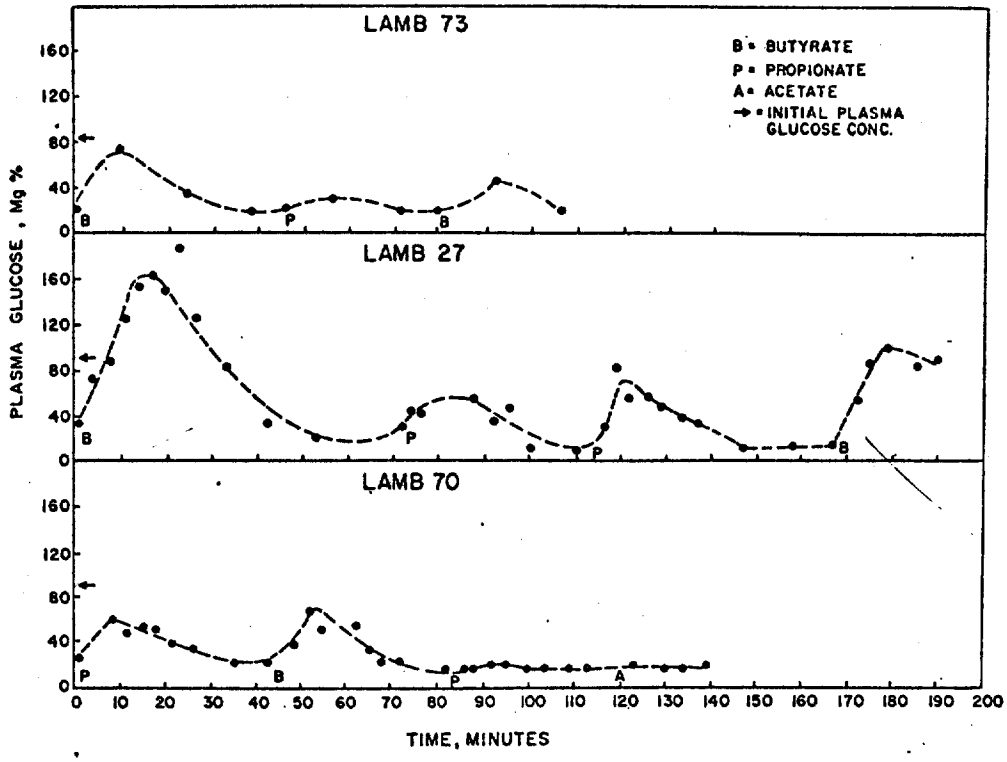

FIG. 1. Plasma glucose concentration in fasted, insulin-induced hypoglycemic lambs after the intravenous infusion of 2·5 mM/kg of the various volatile fatty acids. The symbols on the graphs, B (butyrate), P (propionate) and A (acetate), indicate the time of fatty acid injection.

Since it had been reported in earlier work (Kronfeld, 1957) that injection of β-hydroxybutyrate was followed by increased blood glucose levels in sheep, two lambs were injected with this ketone body and the response compared to that evoked by an equimolar amount of butyrate. As shown in Fig. 3, injection of β-hydroxybutyrate had no effect in either lamb, although injection of butyrate before or after the β-hydroxybutyrate caused a marked increase in plasma glucose

concentration. These results indicate that the effect of butyrate on plasma glucose concentration is not mediated by the β-hydroxybutyrate formed in butyrate metabolism.

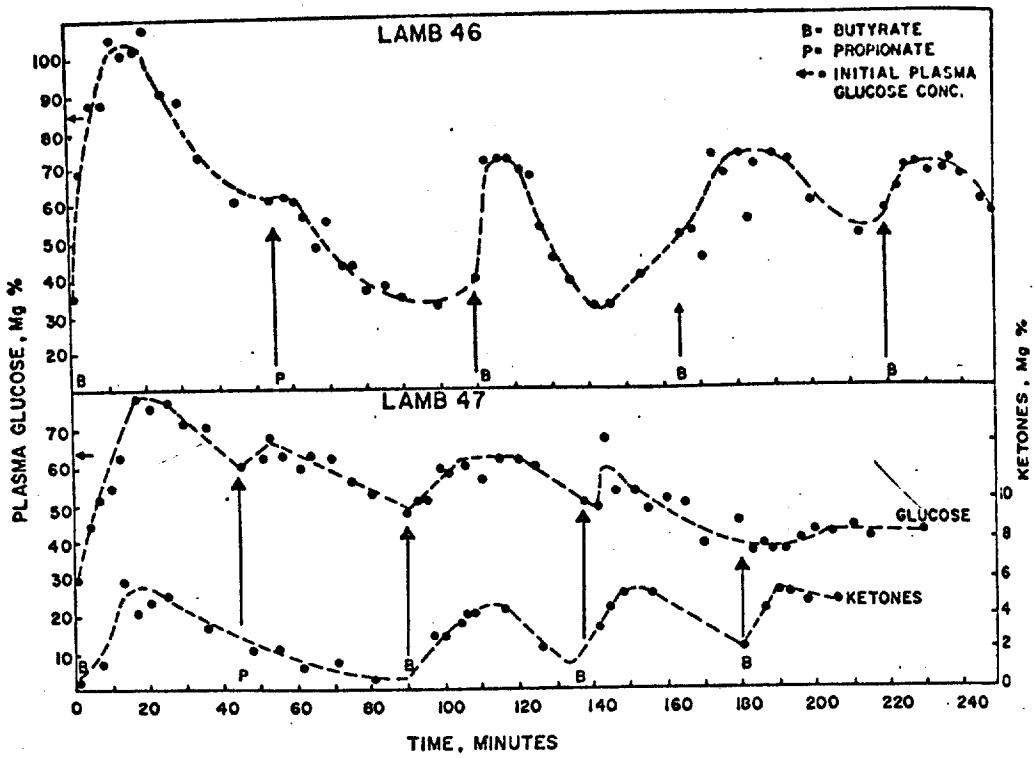

FIG. 2. Plasma glucose and total ketone body concentration in fasted, insulin-induced hypoglycemic lambs after repeated injections of butyrate and propionate at the rate of 2·5 mM/kg.

The discrepancy between the present results and those reported earlier (Kronfeld, 1957) may have been due to differences in experimental procedure. The lambs used in the present study had venous catheters implanted at least 24 hr prior to the experimental period. This technique allows frequent and rapid blood sampling or injection of metabolites with minimum disturbance to the animal. In contrast, sampling of blood by hypodermic puncture of the vessels would tend to excite the animals and it would be impossible to distinguish a hyperglycemic response to an injected metabolite from that caused indirectly *via* the sympathetic nervous system, due to animal excitement.

In all of the preceding experiments the lambs were held until they were in severe insulin-induced hypoglycemia, either stage 3 or 4 of Reid (1951), before the volatile fatty acids were injected. Invariably, the injection of either propionate or butyrate led to rapid recovery and within 10–15 min the lambs appeared

completely normal (see Figs. 4–7); however, they would lapse back into the hypoglycemic convulsions 45–60 min post-injection. The return to stage 3–4 hypoglycemia was quite rapid, occurring in less than 10 min from the first indication of depression. The relapses were caused by the continuing effect of the insulin originally injected to initiate the hypoglycemia. When the experiment was terminated, the lambs were injected with glucose (2·5 mM/kg) and given food. They were then able to maintain plasma glucose levels without recurrence of convulsions.

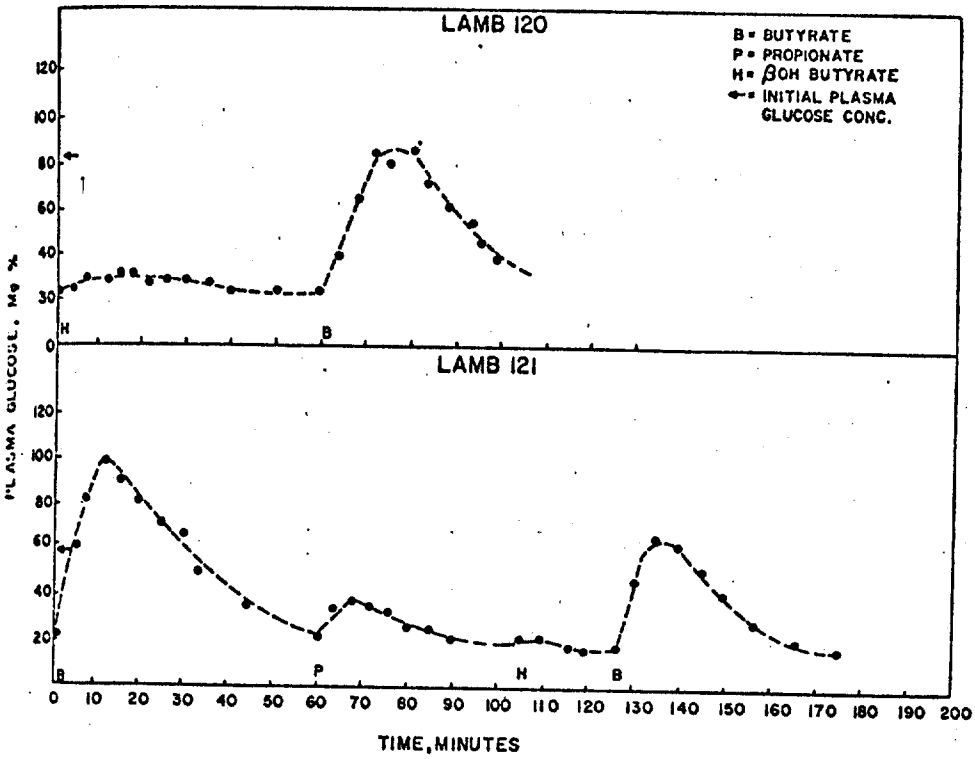

FIG. 3. Plasma glucose concentration in fasted, insulin-induced hypoglycemic lambs after intravenous injection of β-hydroxybutyrate and volatile fatty acids at a rate of 2·5 mM/kg.

Three young calves were used in parallel experiments to measure the response to butyrate in another ruminant species. The results, summarized in Fig. 8, show that in only one of the calves was there a definite increase in plasma glucose concentration and this was modest, about 25 mg per cent compared to the response seen in lambs, 76 mg per cent increase, on the average, following the first butyrate injection. Even though the injected volatile fatty acids in calves did not result in dramatic changes in plasma glucose concentration as seen in lambs, butyrate and propionate did cause the same marked remission of hypoglycemic convulsions. In all three calves, only 10–15 min elapsed following butyrate or propionate

injections until the animals were able to stand and walk about and appeared completely normal; as with the lambs, the calves relapsed into stage 3 or 4 of hypoglycemia (Reid, 1951) in 45–60 min and it was necessary to inject intravenously with glucose and offer feed to interrupt the recurrent convulsions. Intravenous injection of acetate had no effect on the hypoglycemic convulsions nor the plasma glucose (Fig. 8) in the calves.

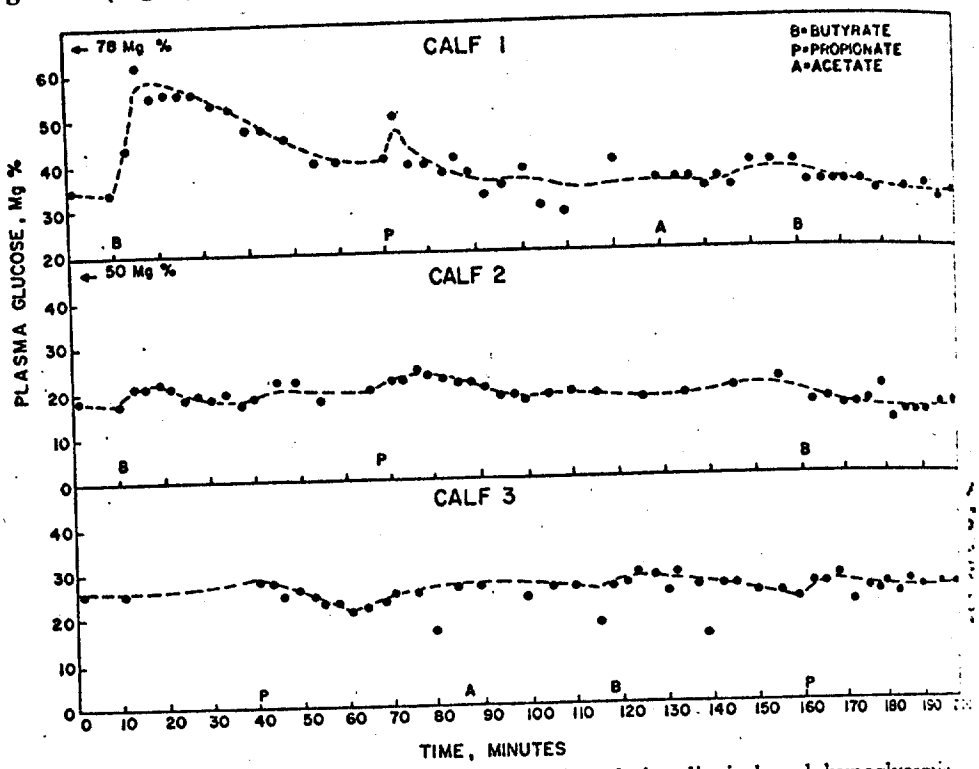

FIG. 8. Plasma glucose concentration in fasted, insulin-induced hypoglycemic calves after the intravenous infusion of 2·5 mM/kg of the various volatile fatty acids. The symbols on the graphs, B (butyrate), P (propionate) and A (acetate), indicate the time of fatty acid injection.

In two dogs and a horse no remission of hypoglycemic depression and very little alteration of the plasma glucose concentration was seen with any of the volatile fatty acids (Table 1).

In an effort to determine what effect the plasma glucose concentration has on the glucogenicity of butyric acid, two 2-week-old nursing lambs were injected with butyrate or acetate ½ hr after the morning nursing when the initial plasma glucose was over 100 mg per cent. The response (see Fig. 9) was essentially the same as seen in the fasted insulin hypoglycemic lambs, butyrate causing an increase in plasma glucose of 60–80 mg per cent while acetate was without effect. These results do not support Kronfeld's hypothesis (1956) that butyrate causes an increase

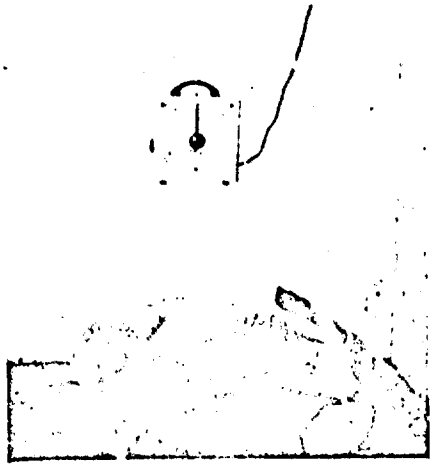

FIG. 4.

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FIG. 6.

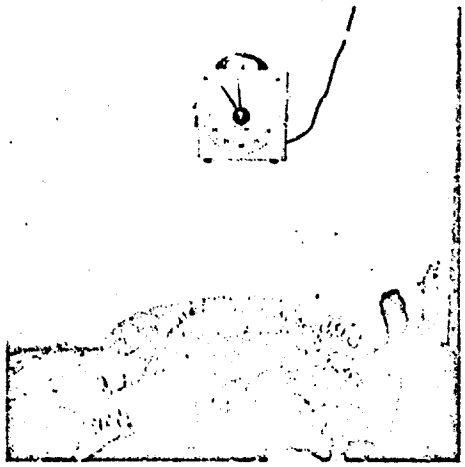

FIG. 7.

FIGS. 4–7. Lamb in hypoglycemic convulsions; butyrate 2·5 mM/kg administered intravenously at time 0. Lamb had responded and was able to stand in 13 min (Fig. 5) and was completely free of hypoglycemic symptoms in 23 min (Fig. 6). Convulsions reoccurred 54 min post butyrate injection (Fig. 7).

of blood glucose when the concentration of the latter is low and a decrease when the concentration is high. In the present study the effect of butyrate was to increase plasma glucose when it was initially either low (Figs. 1–3) or high (Fig. 9).

TABLE 1—EFFECT OF INTRAVENOUS INJECTION OF VOLATILE FATTY ACIDS ON PLASMA GLUCOSE CONCENTRATION IN NON-RUMINANT ANIMALS

	Maximum plasma glucose increase in mg % after volatile fatty acid infusion		
Animal	Acetate	Propionate	Butyrate
Dog 1	0	5	6
Dog 2	0	7	16
Horse	–	10	0

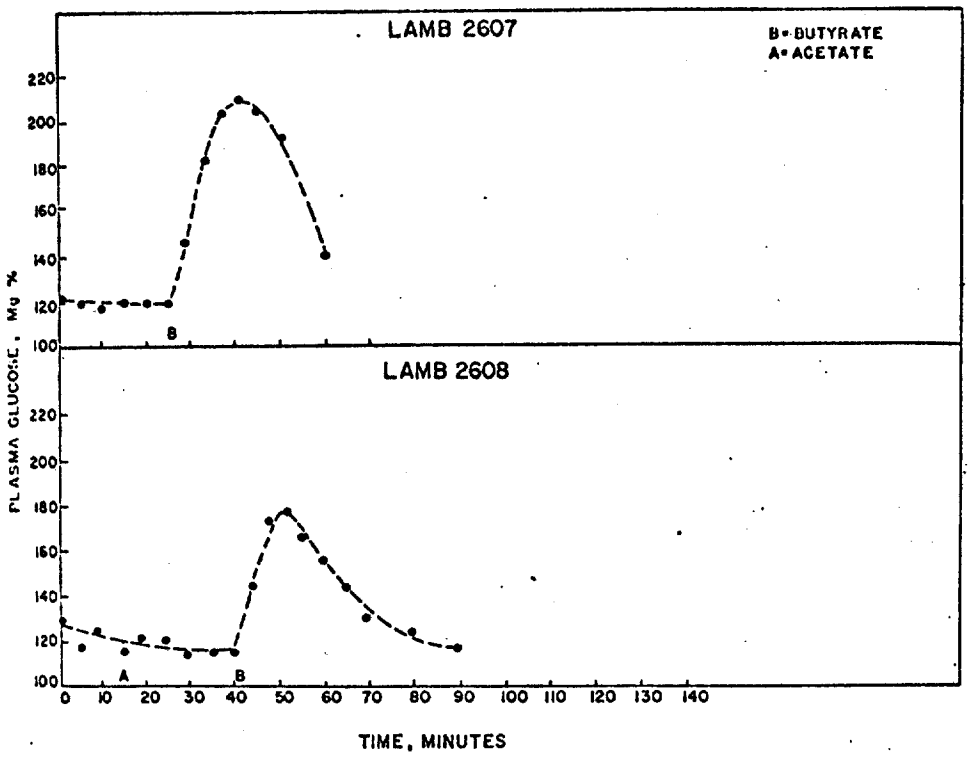

FIG. 9. Plasma glucose concentration in normal fed lambs after the intravenous injection of 2·5 mM/kg of butyrate and acetate.

It has been shown that the injection of butyric acid can cause an activation of liver phosphorylase in the intact and adrenalectomized animal (Phillips *et al.*, 1965). This response was not observed in studies with liver slices (Ash *et al.*, 1964).

In an effort to clarify the relationship between plasma butyrate concentration and liver response three *in situ* liver perfusions were performed on lambs. The plasma glucose concentration, liver glycogen concentration and liver phosphorylase activity were measured at various times during the perfusion. The results, shown in Fig. 10, were variable presumably due to experimental conditions, sampling

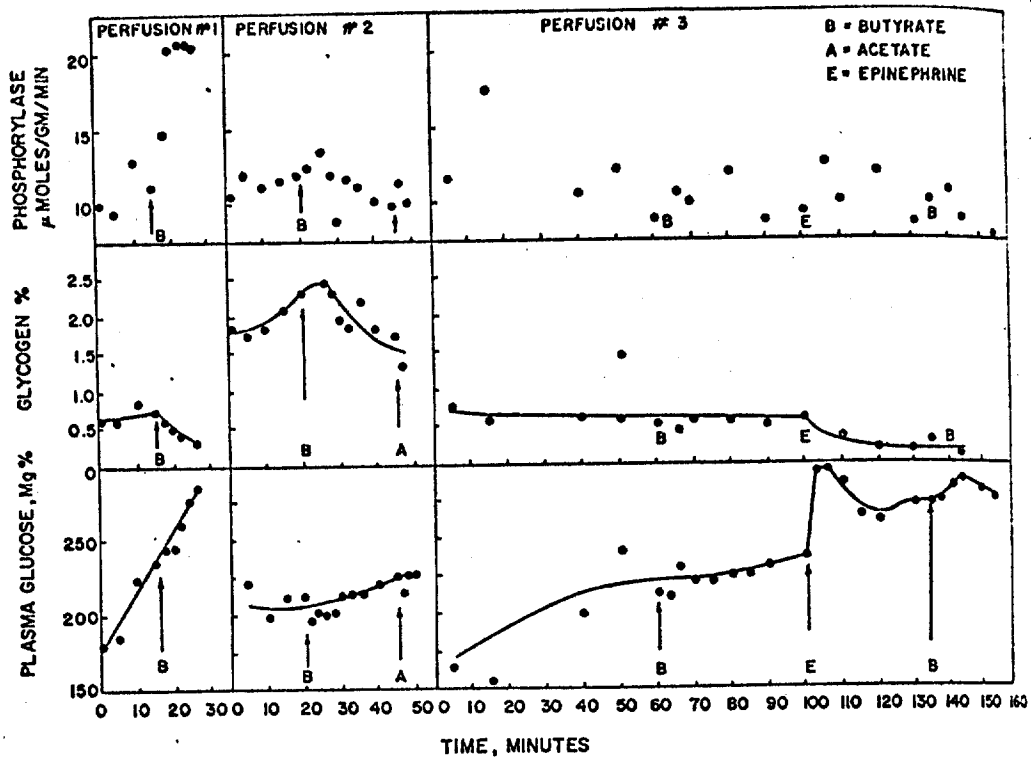

FIG. 10. Plasma glucose concentration, liver glycogen concentration and liver phosphorylase activity during *in situ* liver perfusions.

problems and bleeding from sample sites. In addition the glucose concentration was increasing rather rapidly in the perfusing medium so that it was difficult to establish an effect of butyrate, under these *in vitro* conditions, on the liver. It appears that butyrate may have caused an increase of phosphrylase in the first infusion but there is no definite evidence of a similar effect in the next two perfusions. In the third perfusion the liver appeared to respond to epinephrine but showed little or no response to butyrate injection.

DISCUSSION

The volatile fatty acids, propionate and butyrate, markedly increased plasma glucose concentration in lambs when injected intravenously in hyperphysiological amounts and, simultaneously, the compounds relieved hypoglycemic convulsion.

It was also possible to relieve such convulsions in calves by volatile fatty acid injection but, in these ruminants, there was no concomitant increase in plasma glucose. This lack of effect of plasma glucose concentration in calves may indicate that the central nervous tissue can utilize butyrate as an energy source, a possibility supported by the fact that cerebral respiratory quotients of less than one have been measured in some ruminants (Davey *et al.*, 1961). On the other hand, butyrate may be utilized by muscle and thereby have a sparing effect on glucose or glucose precursors which provide substrate for the brain. Whatever the response to butyrate it seems restricted to ruminants as it was not evident in limited trials using either the horse, another herbivore, or the dog, a carnivore. The fact that neither of these species is normally metabolically dependent on volatile fatty acids may account for the lack of response. It is also apparent that the increase in plasma glucose concentration following injection of butyrate does not depend on existing glucose concentration, as had been suggested (Kronfeld, 1956), but can occur in sheep with normal blood sugar levels (Ash *et al.*, 1961) or in lambs under conditions of relative hyperglycemia, as shown in Fig. 9.

The hyperglycemic response to butyrate in the intact lamb involves, at least in part, release of glucose from the liver. In the normal or adrenalectomized lambs, injection of butyrate was followed by increased levels of active phosphorylase, decreased levels of liver glycogen and hyperglycemia (Phillips *et al.*, 1965). The response in adrenalectomized lambs eliminated epinephrine as a factor in the increased plasma glucose concentration but the possibility remained that some other factor, such as glucagon, may mediate the changes seen after butyrate injection. An effort was made to eliminate glucagon as a factor by perfusing lamb livers *in situ*. The results from these perfusions were too variable to permit valid conclusions so the possibility remains that butyrate may have an indirect effect on liver glycogenolysis mediated by glucagon (Lukens, 1959). It is also possible that glycogenolysis is so sensitive to environmental conditions that anoxia (Morgan & Parmeggiani, 1964), which is difficult to avoid in perfused organs, or some other condition may have effectively masked the response to butyrate during the perfusion.

The possibility of a direct conversion of butyrate to glucose (Kronfeld, 1957) seems unlikely in view of the decreasing magnitude of the response with repeated injections. Studies in ruminants with C^{14}-butyrate have also indicated that pathways for direct conversion of butyrate to glucose do not occur in the intact cow or sheep (Black *et al.*, 1961; Annison *et al.*, 1963).

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THE BIOSYNTHESIS OF MILK-FAT IN THE INTACT COW*

by

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INTRODUCTION

Recent publications from our laboratory have been concerned with the roles of the short-chain fatty acids, carbonate and glucose as precursors of milk-constituents[1-9]. These have been studied by the intravenous injection of the various metabolites appropriately labeled with ^{14}C.

The appreciation of the importance of the short-chain fatty acids in ruminant digestion[10] has served to focus attention on the nature of fat synthesis in ruminants. The milk-triglycerides of these animals are characterized by their content of short-chain fatty acids. Reports by POPJAK, FOLLEY, FRENCH *et al.* have thrown much light on the roles of acetate and glucose in ruminant fat synthesis, and this work is extensively reviewed by POPJAK[11] and FOLLEY[12]. Some of their work was conducted on intact goats, but most of the data are from perfused udder and tissue-slice studies. The present report supplements previous knowledge of the utilization of acetate and glucose by observations made on the intact cow. In addition, it provides some information about fat synthesis from carbonate, formate, propionate and butyrate.

METHODS

The equipment and techniques for the conduction of tracer trials have been described by KLEIBER AND EDICK[1]. Following the injection of a ^{14}C-labeled metabolite, continuous samples of respired carbon dioxide were taken for the first 3 hours and then for short periods at intervals up to 2[illegible] hours. Blood samples were taken at half-hour intervals during the first 3 hours, and subsequently at milking times which were 3, 10, 22, 34 and 48 hours after the injection.

In this preliminary survey, the radio-activity of the fat constituents was determined following the injection of each of the metabolites listed in Table I. The table also gives the dosage injected and characteristics of the cows which were used. Individual tracer trials are identified by the name of the metabolite injected, followed by a Roman numeral which indicates the number of the trial in the series carried out with that metabolite.

The fats were separated from each of the milk-samples by the method of ROESE AND GOTTLIEB[13]. Four crude fractions were separated from each fat-sample. These were the glycerol, the water-soluble steam-volatile fatty acids, the water-insoluble steam-volatile fatty acids, and the non-volatile fatty acids. The latter three fractions are each composed chiefly of the C_4, the C_6–C_{10}, and the C_{12}–C_{22} fatty acids respectively. The fractionation procedure is outlined below.

1. 10 g of fat were saponified for 30 minutes with methanolic potassium hydroxide.
2. The non-saponifiable fraction was extracted with ether and discarded.
3. The soap solution was acidified with sulfuric acid and steam-distilled for 2 hours.

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TABLE I

Trial No.	Body weight of cow (kg)	Daily fat production (g)	Position of label	Dosage μc/kg
Carbonate VI	484	542	1-C	20.70
Carbonate XIV	443	576	1-C	23.30
Glucose II	526	971	U-C	2.76
Acetate VII	609	525	1-C	5.72
Acetate X	470	433	2-C	8.20
Propionate II	494	384	1-C	9.31
Propionate IV	545	336	2-C	8.80
Butyrate II	547	302	1-C	7.30
Butyrate IV	450	783	2-C	11.10
Formate II	454	763	1-C	11.0

4. The non-volatile fatty acids were removed from the residue by filtration and ether extraction.

5. The residue was then neutralized with potassium hydroxide and evaporated to dryness. The glycerol was separated from the potassium sulfate crystals with methanol.

6. The steam-distillate was similarly neutralized and evaporated to dryness. It was then redissolved in 10 ml of water, and when this was acidified, the water-insoluble volatile fatty acids came out of solution. They were separated by filtration at 3°.

7. The radio-activity of the fat constituents was measured in the fashion described by KLEIBER[1]. A small amount of each sample was combusted and the resultant carbon dioxide was trapped in barium hydroxide solution, which precipitated barium carbonate. This was planchetted and counted at infinite thickness in a flow-gas counter.

RESULTS

The results are summarized in Table II. The radio-activity of each sample was determined as the "Standardized Specific Activity", of which the units are μc/gram atom carbon: μc injected/kg body weight. By dividing the specific activity by the administered dosage, it is possible to compare results from experiments in which different dosages were injected into animals of different weights. Owing to the different

TABLE II

SUMMARY OF DATA FROM ALL TRIALS

Trial	Position of label	$\Sigma_0^{48} \lambda s \Delta t$ for			% of activity derived via carbonate pool			% of dose appearing in each of				$\int_0^{48} \rho s\, dt$ Resp. CO_2
		Lactose*	Glycerol	Fatty acids	Lactose*	Glycerol	Fatty acids	Lactose*	Glycerol	Fatty acids	Total* milk	
Carbonate VI	—	6.9	4.4	1.0	—	—	—	1.0	0.07	0.26	1.6	60.9
Carbonate XIV	—	9.7	6.6	0.6	—	—	—	1.3	0.14	0.18	2.0	87.1
Glucose II	Random	28.7	31.6	2.6	13.4	0.8	13.5	55.4	9.4	1.0	71.6	34.4
Acetate VII	1-C	10.3	5.0	25.3	86.9	100.0	3.0	2.0	0.1	4.6	6.8	74.6
Acetate X	2-C	29.5	30.2	26.5	25.5	15.4	2.4	3.4	0.4	4.6	9.0	62.8
Propionate II	1-C	23.5	26.7	6.6	54.6	30.0	16.0	3.9	0.3	1.0	7.0	106.9
Propionate IV	2-C	64.0	50.4	4.5	8.0	7.0	10.0	6.2	0.5	0.7	9.3	46.3
Butyrate II	1-C	15.1	8.3	10.6	56.1	62.0	7.0	2.0	0.7	1.2	4.7	70.6
Butyrate IV	2-C	27.7	25.3	7.2	22.6	16.0	7.0	6.1	0.8	2.9	17.2	52.1
Formate	—	20.5	9.4	0.5	22.1	30.0	75.0	4.0	0.2	0.16	12.4	37.7

* These values were determined in previously reported work, and are included in this table for comparison with the values for glycerol.

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rates of metabolic processes, the radio-activity of a single sample is not the activity of that constituent as synthesized at that instant, but is rather the average radio-activity for the time-interval since the last milking. However, as milk-constituents are stored by the cow in the udder as soon as they are produced, and are thenceforth presumably metabolically inert, the radio-activity of the sample multiplied by the time interval between milkings gives the time-integral of the radio-activity. These integrals can be summated through a 48 hour period (at the end of which there is no further appreciable radio-activity in any milk-constituents). Thus, in Fig. 1, the straight line forming the top of each block is the average radio-activity of that milk-constituent during that period of time. The area of the block (down to the abcissa) is the time-integral of the radio-activity for that milking, and the sum of the areas of all the blocks is the total time integral. This last value is the best indication of the extent to which the milk-constituent in question has been formed from the radio-active precursor. A dotted line in Fig. 1 is an estimate of the locus of the instantaneous activity of the glycerol moiety of the milk-fat. It should be noted however, that this is just one of a family of curves that could be drawn to fit the experimentally determined time-integrals. Similar graphs can be drawn of the results in each of the tracer trials conducted.

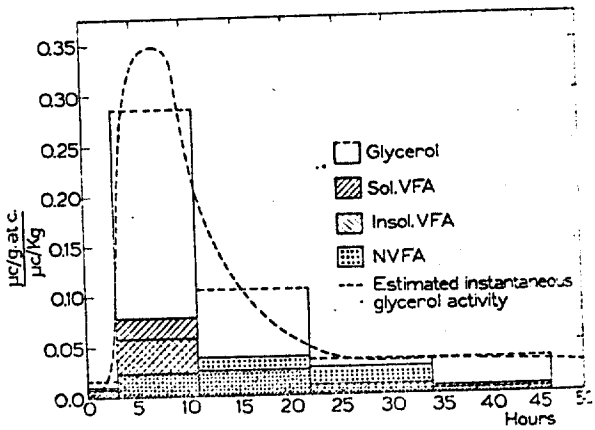

Fig. 1. Standardized specific activities following injection of ^{14}C bicarbonate.

Bicarbonate trials

It will be noted that the time-integrals of the radio-activities of the fat-moieties following the injection of $NaH^{14}CO_3$ were of a low order of magnitude (Table III). The results from these two trials are in close agreement, which suggests that the methods are reliable.

The extent of carbon dioxide fixation into any milk-constituent is conveniently expressed by KLEIBER[2] as the "Transfer Quotient"*. In all trials samples of respired carbon dioxide are taken throughout the experimental period, and the standardized specific radio-activities can be plotted to give a smooth curve. Measurements have shown that the radio-activity of the respired carbon dioxide is the same as that of the plasma bicarbonate, which is assumed to be that of the tissue fluid in general. The curve is integrated over a 48 hour period, and similarly the radio-activity of each milk-constituent is integrated over the same period. When the integral of the milk-constituent radio-activity is divided by that of the carbon dioxide, the quotient,

* Transfer quotient "q" $= \dfrac{\Sigma_0^{48} \lambda s \, \Delta t}{\int_0^{48} \varrho s \, dt}$ where λs is the standardized specific activity of the milk-constituent, and ϱs is that for the respired carbon dioxide.

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"q", expresses the relative transfer of carbonate carbon to the milk-constituent concerned.

Calculations from Carbonate trials VI and XIV give transfer quotients of carbonate carbon to glycerol of 0.0725 and 0.0760 respectively. 7.4% is taken as the average. Once determined in this fashion, the transfer-quotient can be applied to calculate the proportion of the activity in a milk-constituent which has come via the carbonate pool in trials where metabolites other than labeled carbonate have been injected. As before, the respired carbon dioxide activity is integrated over a 48 hour period, and this integral multiplied by "q" is divided by the integrated radio-activity of the milk-constituent,

$$\text{i.e. } \frac{q \times \int_0^{48} \varrho s \, dt}{\sum_0^{48} \lambda s \, . \, \Delta t} = \text{transfer of carbonate carbon to milk constituent.}$$

The values obtained from these calculations are listed in Table II.

The transfer quotient of carbonate carbon to the fatty acids of milk fat was 0.017 and 0.007 in Carbonate trials VI and XIV respectively. This indicates an average of about 1.0%, which is almost negligible.

Glucose trial

The glycerol following the injection of ^{14}C-labeled glucose was extremely radio-active, and was in fact the most radio-active compound isolated from the fats in this series of experiments. Conversely, the radio-activities of the fatty acids were very low. Table II shows that 71% of the injected isotope was recovered in the milk, which is greater by a factor of 4 than the isotope recovery in the milk following the injection of any of the other metabolites studied. The radio-activity of the respired carbon dioxide was correspondingly low.

Acetate trials

After the injection of either 1-^{14}C or 2-^{14}C labeled acetate, the percentage of the isotope appearing in the milk was low compared with that in the glucose trial, and the respired carbon dioxide radio-activity was correspondingly greater. The degree of labeling in the fatty acid moieties was about the same, regardless of the position of the label on the injected acetate. It will be noted however, that the injection of 1-^{14}C-labeled acetate gave rise to practically no radio-activity in the glycerol, and that such radio-activity as was observed could be accounted for via the fixation of carbonate carbon. On the other hand, the 2-^{14}C-labeled acetate injection yielded glycerol as radio-active as the non-volatile fatty acid fraction.

Propionate trials

Our results show that, as might be expected, propionate is a potent precursor of glycerol. The time-integral of the specific radio-activity of the glycerol was higher than that of the fatty acids.

Butyrate trials

It is apparent that butyrate is intermediate between the extremes of lipogenic and glycogenic potentialities exhibited by acetate and propionate.

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Formate trial

Formate is a poor precursor of fatty acids, and 75% of the small activity observed in the latter after injection of labeled formate was derived via the carbonate pool. The glycerol radio-activity was considerably higher than that of the fatty acids, but still low when compared with the glycerol from all other trials except the Carbonate trials.

Delay in fat secretion

In each of these trials, (with the exception of that with labeled glucose), the most highly radio-active fat obtained was that from the second milking (11 hours). This is in contrast with the casein and lactose, which always reached their highest radio-activity in the first milk sample (3 hours). In the case of the labeled glucose injection, the fat radio-activity was the same in the first and second samples.

DISCUSSION

Fatty acid synthesis

The potency of acetate as a milk-fat fatty acid precursor, and the large amount of acetate available to the cow, leave little doubt that it is quantitatively the main source of this moiety of the milk-fat. In our investigation, the time-integrals of the radio-activities of the milk-fat fatty acids after the injection of 1-^{14}C and 2-^{14}C-labeled acetate were about the same. Also, the percentage of the injected isotope which appeared in the fatty acids was the same in either case. These similarities suggest that acetate is utilized as a two-carbon unit for fatty acid synthesis, which is of course, consistent with the present concepts.

The radio-activity of the milk-fat fatty acids is smaller after injections of labeled butyrate than after those of correspondingly labeled acetate. That is to say, a smaller part of the blood butyrate than of acetate is used for fatty acid synthesis. In each milk-fat sample taken after labeled butyrate injections, the radio-activity of the soluble volatile fatty acids is higher than that of the insoluble volatile fatty acids in the same milk-fat sample (Table III). After acetate injections, however, the soluble volatile fatty acids are less radio-active than the insoluble volatile fatty acids. As

TABLE III

COMPARISON OF THE PATTERN OF LABELING IN THE THREE FATTY ACID FRACTIONS FROM SUCCESSIVE MILK-FAT SAMPLES*

Only the results after 1-^{14}C acetate and butyrate injections are compared here, but the pattern of labeling is essentially the same after the 2-^{14}C injections. The units are μc/gram atom C: μc/kg body weight.

Milk-sample number	1-^{14}C acetate injection			1-^{14}C butyrate injection		
	Sol. VFA	Insol. VFA	NVFA	Sol. VFA	Insol. VFA	NVFA
1	4.7	5.0	1.2	0.3	0.3	0.04
2	4.9	5.7	1.4	3.4	2.6	0.34
3	1.7	2.1	0.5	1.7	1.4	0.10
4	1.0	1.4	0.3	0.5	0.2	0.05
5	0.3	0.5	0.1	0.3	0.1	0.04
$\Sigma_0^{48} \lambda s/At$	61.6	77.8	17.8	53.2	38.0	5.7

* VFA = volatile fatty acids.
NVFA = non-volatile fatty acids.

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the soluble volatile fatty acid fraction is almost entirely butyric acid, it seems likely that some blood butyrate is incorporated directly into the butyric acid moiety of the milk-fat, and that not all blood butyrate is broken down into two-carbon units.

The similarity of the time-integrals of the standardized specific activities of the fatty acids after the injection of both 1-^{14}C and 2-^{14}C-labeled butyrate indicate that for most of the synthesis of fatty acids two carbon units are the smallest split products of butyrate.

The time-integrals of the standardized specific activities of the fatty acids from all trials listed in Table II, show that none of the other metabolites investigated is a potent precursor of milk-fat fatty acids. In particular, the low activity of the fatty acids following the injection of labeled glucose is in interesting contrast to the expected result in a non-ruminant. In a non-ruminant which is synthesising fat from a mainly non-fat diet, the necessary acetyl CoA units are derived from glucose, so that the injection of labeled glucose would give rise to highly labeled fatty acids in the fat[11]. In view of the fact that the cow absorbs little or no glucose from its gut[10], and that its blood glucose is synthesized from rumen fatty acids, it is hardly surprising that it does not use glucose as a source of fatty acids for milk-fat synthesis.

The level of radio-activity in the fatty acids after the injection of labeled propionate is similar to that after the injection of labeled glucose. There are undoubtedly pathways whereby propionate carbon is incorporated into fatty acids, but our data suggest that these are relatively unimportant in the cow. This is probably because of the large demand on blood propionate for glycogenesis.

The transfer quotient of carbonate carbon to fatty acids is only about 1%. This low value is of interest because a metabolic reaction of carbon dioxide with acetone to yield aceto-acetic acid has been demonstrated[14]. The aceto-acetate could be a precursor of fatty acids, but our results on carbonate transfer to fatty acids show that this pathway is either absent or negligible in the cow.

Formate too is a poor precursor of fatty acids, and can be considered as unimportant in this phase of the cow's metabolism.

Glycerol synthesis

DOERSCHUK has studied glycerol metabolism in rats[15], and an accepted pathway of glycerol synthesis is that from dihydroxy-acetone phosphate produced in glycolysis. The extremely high glycerol radio-activity after the injection of labeled glucose into a cow suggests that a similar pathway is operative in this case. FOLLEY[16] found that ruminant mammary gland slices will synthesize fat from a substrate containing acetate as the sole organic constituent. The fat synthesis was enhanced by the addition of glucose, but only to the same extent as by the addition of glycerol. He also found that non-ruminant mammary gland slices would not synthesize fat from acetate, with or without glycerol, unless glucose was also present. FOLLEY's interpretation of these observations is that the main function of glucose in ruminant fat synthesis is as a precursor of glycerol, and that it is not required as a source of energy for the synthetic process. However, glucose cannot be regarded as a "Prime precursor", because in the cow, the glucose itself must be synthesised from fatty acids. Of the metabolites available from the rumen, our data show that propionate is the most potent precursor of glycerol. This observation concurs with that of KLEIBER *et al.*[5,7] and of BAXTER[8] regarding the synthesis of lactose.

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The injection of either labeled acetate or butyrate gave rise to radio-active glycerol. Therefore, all three of the principal rumen fatty acids are precursors of the glycerol moiety of the milk-fat. A glycogenic pathway in each of these three fatty acids seems to be via the tricarboxylic acid cycle. In each case, the ^{14}C activity in glycerol after the injection of the 1-^{14}C-labeled fatty acid is much less than after the injection of the same fatty acid labeled in the 2-C position. This dissimilarity would be expected of a fatty acid involved in the Krebs cycle, because a label on the carboxyl group of the injected fatty acid would appear on a terminal carbon of succinate, where it would be immediately vulnerable to oxidation. There is a greater chance of a 2-^{14}C label escaping the oxidation to CO_2 in the tricarboxylic acid cycle.

In view of the different glycerol radio-activities following the injections of 1-^{14}C and 2-^{14}C-labeled butyrate, and of what is known about β-oxidation of fatty acids in general, it seems likely that some butyrate is split into two acetyl CoA units. These could be a source of glycerol and glucose via the tricarboxylic acid cycle when it is operating towards a net synthesis... as it appears to in the cow. However, as discussed by KLEIBER[22], labeled butyrate gives rise to lactose of greater activity than does labeled acetate, which suggests that butyrate may be a precursor of glucose without passing through the acetate pool.

The possibility of a glycogenic pathway for butyrate which does not pass through the acetate pool is further indicated by two observations related to the data reported here.

1. The percentages of glycerol carbon that could be derived via the carbonate pool after the injection of 1-^{14}C and 2-^{14}C-labeled acetate were 100% and 87% respectively. The corresponding figures for butyrate injections are 62% and 16% (Table II).

2. As discussed under *Fatty acid synthesis*, the relative radio-activities of the soluble and insoluble volatile fatty adid fractions of the milk-fat after the injections of labeled butyrate suggest that some of the butyrate is not broken down to acetyl CoA units. If the only glycogenic pathway of butyrate were via the acetate pool, then butyrate would be no more glycogenic than acetate; whereas in fact, it is.

The study of glycogenesis from propionate has been a very active field. A pathway via acrylate to lactate and pyruvate has been demonstrated in rats[17], but our results in the cow are not consistent with this pathway. If it were important in the cow, the glycerol (and lactose) radio-activities after the injection of 1-^{14}C and 2-^{14}C-labeled propionate would be the same. Table II shows that this is not so. The main evidence for the rejection of this pathway (in cow metabolism) is provided by BLACK[9], who has studied the casein amino-acids from the same trials that are discussed in this report. In the propionate trials, BLACK found that the pattern of labeling in the alanine (which reflects that of pyruvate) was inconsistent with the acrylate pathway. A previously postulated pathway in which propionate enters the Krebs cycle via condensation with carbon dioxide to give succinate has been demonstrated by FLAVIN[18]. This pathway is consistent with our results, and would explain in part the large amount of carbon dioxide fixation observed in glycogenesis in the cow.

In the glycogenesis from acetate, propionate and butyrate, the fixation of carbon dioxide is an important factor, as is shown by the high transfer via carbonate pool listed in Table II. As these three fatty acids are quantitatively the most important products of the cow's digestive system, it follows that carbon dioxide fixation

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is very important in the overall synthesis of the glycerol moiety of milk-fat.

The glycerol radio-activity following the injection of labeled formate is low (Table II). One of the known reactions of formate is that with glycine to yield serine[19]; serine can be deaminated to yield pyruvate[20], and then pyruvate is a source of glycerol. BLACK[21] has found that the β-carbon of serine (in the casein) is highly radio-active after the injection of labeled formate. Therefore in the cow glycerol might be derived from formate via an amino acid. The degree of radio-activity in the glycerol after the injection of labeled formate, however, shows that this unusual pathway is quantitatively unimportant.

In most cases, the radio-activity of the glycerol was found to be the same as that of the lactose which had been determined in earlier work[7,8]. However, in two of the trials, acetate VII and butyrate II, the glycerol radio-activity time-integral was only about one half of that of the lactose. Both of these trials involved the injection of a 1-^{14}C-labeled fatty acid, but there does not seem to be any ready explanation of the difference.

Secretion of milk-fat

Very little is known about the mechanism of milk-fat secretion, but the long-standing observation that the "Strippings" from a cow have a higher than average fat-content would suggest that at least some of the fat is not secreted until the time of milking. It is not known whether this is the result of pressure conditions within the udder, or of some possible change in hormonal status when letting down the milk. The older theory that the "cream rose to the top" of the udder has been rejected on several grounds[22].

In our series of experiments, it has been strikingly apparent that fat is secreted with a greater delay than are the casein and lactose components. The delay is more than 3 hours and less 10 hours, but the data do not permit an exact calculation of the length of the delay. The rapidity of fat synthesis *in vitro*[16] precludes the possibility that the delay is due to low reaction rates in the enzymic processes of synthesis. A comparison of the radio-activities of fat-components with those of other milk-constituents suggests that the fat obtained in the second milk-sample was actually synthesized during the first milking period. The delay in the peak of radio-activity is apparent in all of the fat constituents, including glycerol, and this shows that the delay occurs after esterification of the fatty acids to neutral fat. It is presumed therefore, that the fat is retained within the cells in which it is synthesized, and that the delay is a delay of the actual secretion into the lumen of the gland.

SUMMARY

The behavior of the principal metabolites of the cow as precursors of milk-fat has been studied. This has been carried out by determining the specific activities of the various fat-constituents after the injection of the metabolites labelled with ^{14}C. Significant fixation of carbon dioxide into the glycerol moiety was observed; that into fatty acids was almost negligible. Glucose is important in fat synthesis as a precursor of glycerol, but fatty acid synthesis from glucose is on a very small scale. Acetate is the most potent precursor of fatty acids, but it is also a source of glycerol. The transfer of acetate carbon to glycerol appears to be by way of the tricarboxylic acid cycle, through both the incorporation of acetyl CoA and by carbon dioxide fixation. Propionate is unimportant in fatty acid synthesis, but the activity of glycerol following the injection of labelled propionate supports the view that propionate is the main carbohydrate precursor in the cow. Butyrate shows intermediate lipogenic and glycogenic functions. Formate is not a source

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of fatty acids in milk-fat, but does contribute some carbon to glycerol. The peak radio-activity in all of the fat-constituents was observed in the second milk-samples (taken at 11 hours), whereas for the casein and lactose it was in the first (3 hours after injection of the tracer).

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FLAVIN STIMULATION OF METHEMOGLOBIN REDUCTION IN CELL-FREE BACTERIAL EXTRACTS*

by

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Although HUENNEKENS AND GABRIO[1] have purified and characterized a methemoglobin reductase from red blood cells, the mechanism of methemoglobin reduction by bacteria still remains unknown. Since the early observations of NEILL AND AVERY[2] and NEILL[3] that Pneumococci and anaerobic bacilli would reduce methemoglobin, the question of whether or not bacteria contain a specific methemoglobin reductase (and if so, why) has been an open one.

The observation in this laboratory that methemoglobin would inhibit lumines-

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Hygienic Normalization of the Content of Propionic Acid and of Its Sodium Salt in Reservoirs

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The technological process of manufacturing synthetic fatty acids (SFA) out of oil paraffins is associated with the formation of sewage, which contains, in its makeup, propionic acid and its sodium salt. The hygienic normalization of the content of these substances, in reservoirs, will make it possible to substantiate the requirements for the construction of purifying equipment, and to control the effectiveness of its operation.

Propionic acid, CH_3CH_2COOH is a highly mobile liquid with an acrid odor; it mixes with water in any ratios. Its sodium salt (sodium propionate) -- CH_3CH_2COONa -- is a white crystalline powder with a weak, characteristic odor, which is readily soluble in water.

Propionic acid and sodium propionate impart to water an aromatic odor and an acidulous-astringent taste. The threshold of perception of the odor (an intensity of 1 point) for propionic acid has been established at a level of 4.9 milligrams/liter; the practical threshold (an intensity of 2 points) -- 11.5 milligrams/liter; and for sodium propionate -- respectively, at a level of 13.7 and 28.9 milligrams/liter. Heating aqueous solutions of both compounds up to 60 deg negligibly enhances the intensity of the odor; boiling, however, eliminates or considerably weakens it. Chlorination of the water, which contains the given preparations, does not bring about any provocation in the odor. Threshold concentrations of propionic acid (the threshold of sensation and the practical threshold), with respect to the taste, have been established at a level of 5.9 and 11.9 milligrams/liter, and that of sodium propionate -- respectively, at a level of 9.9 and 21.4 milligrams/liter.

The stability of the given preparations, in water, was determined by means of indirect methods -- according to the odor and taste, and through a test for the survival rate of Daphnia (water flea). Investigations have shown that propionic acid, and to a still greater extent, sodium propionate, represent compounds of low stability.

We began a study of their effect on the overall sanitary precedures in reservoirs starting with experiments on the biochemical consumption of oxygen (BCO). It has been established that propionic acid, in concentrations of up to 0.1 milligrams/liter, and sodium propionate, in concentrations of up to 0.3 milligrams/liter, have practically no effect on the BCO. In samples containing the indicated preparations ranging from 0.1 - 0.3 to 15 milligrams/liter, there is noted an increase in the BCO from the first days of the experiment. At the same time, there has been disclosed a direct dependence of the strengthening of the intensity of the BCO processes on an increase in the concentration of the substances under study in water. Thus, after 5 days of incubation, the BCO grows in the samples with 1 milligram/liter of propionic acid or sodium propionate by 17 - 20%, and in samples with 5 milligrams/liter, by as much as 83 - 96%, in comparison with the control.

At the present time, during the hygienic normalization of substances that raise the level of oxygen consumption, it is necessary to establish, certainly, the oxygen consumption for a unit, by weight, of the substance, in completing the carbonic phase of oxidation, and also, to determine the rate constant for the oxidation of the substance (S. N. Cherkinskii and S. F. Fridland). Judging from the results of the investigations, with concentrations of propionic acid and its sodium salt equal to 1 - 5 milligrams/liter, the magnitude of the rate constant for the consumption of oxygen will fluctuate within the range of 0.1 - 0.17; this attests to oxidation at the same rate as oxidation of domestic sewage.

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The time for the practical (99%) termination of the oxidation process of the given substances, in water, we established in accordance with the formula deduced by V. M. Prusakov. From a calculation for the BCO magnitude, as permissible in accordance with the public health regulations, for a complete 1 milligram of the substances under study, it follows that concentrations of propionic acid and of sodium propionate of 2.5 milligrams/liter are threshold ones for reservoirs of the first type, and 5 milligrams/liter for reservoirs of the second type. However, these concentrations increase the BCO, respectively, by 40 - 50 and 80 - 90 % in comparison with the control. In connection with this, for the purpose of attaining a greater sanitary reliability, the threshold permissible concentration of propionic acid and of its sodium salt should be taken at a level of 1 milligram/liter; this brings about an increase in the BCO of no more than 15 - 20%.

In the literature there is no information with respect to the toxicity of propionic acid for warm-blooded animals. As regards sodium propionate, there is a description of only the toxicity of its commercial sample (Khara Saburo and coauthors). We made a study of the toxicological characteristic of propionic acid and of its sodium salt under conditions of acute, subacute, and chronic prophylactic-toxicological experiments. The clinical picture of acute poisoning by the indicated preparations was characterized by a short-term period of excitation (irritation), which is replaced by a prolonged, depressed state of the animal, dyspnea, cyanosis of the ears and extremities. The data of the acute experiment, which had been carried out on white mice and white rats, were treated by the statistical method of probitanalysis and modification of V. B. Prezorovskii's. The LD_{50} of the substances for rabbits was established according to the method of Doikhman and Le Blanc (see table).

Toxicity of Propionic Acid and of Sodium Propionate for Warm-Blooded Animals Under Conditions of Acute Experimentation

(in grams/kilogram)

Substance	White Mice	White Rats	Rabbits
Propionic Acid	1.37 ± 0.15	1.51 ± 0.25	1.9
Sodium Propionate	7.8 ± 0.6	8.15 ± 0.91	6.75

For the purpose of judging the cumulative properties of the preparations, there was carried out a subacute 20-day experiment; In the course of the latter, the white rats received, separately, propionic acid and sodium propionate, figuring, respectively, onl/10 and 1/5 LD_{50}. On the 21st day of the experiment, there was administered a dose of the substances that was equal to a mean-lethal one. The death of the animals had its onset in less than 50% of the cases.

In a 2-month subacute experiment, against a background of the toxic action of the indicated preparations, in a dose equal to 1/20 LD_{50}, it was established that the most essential is the reduction in the level of the sulphhydryl (SH) groups in the serum and in the whole blood of the animals. In the white rats, which had received propionic acid in a dose equal to 1/10 LD_{50}, there was noted a retardation in growth and a reduction in weight, a diminution in the amount of erythrocytes and in the content of hemoglobin in the blood, and an increase in the gravimetric coefficient of the liver. In a chronic prophylactic-toxicological experiment on white male rats, over a period of 6 months, tests were made on doses of propionic acid amounting to 1, 10 and 50 milligrams/kilogram, and on those of sodium propionate amounting to 5, 100 and 400 milligrams/kilogram. An investigation was carried out on the change in weight of the animals, on the content of reticulocytes in the blood, on the sulphhydryl groups in the blood serum, and on the state of the conditioned-reflex activity of the animals; the internal organs of the

animals were subjected to histological examination.

The results of the investigations make it possible to consider the doses of propionic acid amounting to 1 milligram/kilogram (20 milligrams/kilogram) (?) ((should be 20 milligrams/liter (?) -- Translator)) and of sodium propionate amounting to 5 milligrams/kilogram (100 milligrams/liter) inoperative according to prophylactic-toxicological indications.

Conclusions

1. Propionic acid and sodium propionate are capable of deteriorating the organoleptic properties of water, imparting to it an aromatic odor and a sourish-astringent taste. The threshold concentration of propionic acid, in reference to influence on the odor of the water, has been established at a level of 4.9 milligrams/liter, and that os sodium propionate, in reference to the effect on the taste -- at a level of 9.9 milligrams/liter.

2. The most essential feature for propionic acid and its sodium salt is their unfavorable effect on the overall sanitary procedure for reservoirs, and in the first place, on the BCO (biochemical consumption of oxygen) processes. The threshold concentration of each substance, relative to the conventional public health indices, has been established at a level of 1 milligram/liter.

3. 1 milligramkilogram (20 milligrams/liter) should be considered an inoperative dose of propionic acid, under conditions of chronic toxicological experimentation, and in the case of sodium propionate -- at a level of 5 milligrams/kilogram (100 milligrams/liter).

4. The limiting indication of danger for normalized (standardized) substances represents the generally accepted public health one. In determining the conditions for draining off sewage, which contains propionic acid and sodium propionate, into open reservoirs, one may recommend their threshold permissible concentration of 1 milligram/liter.

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ГИГИЕНИЧЕСКОЕ НОРМИРОВАНИЕ СОДЕРЖАНИЯ ПРОПИОНОВОЙ КИСЛОТЫ И ЕЕ НАТРИЕВОЙ СОЛИ В ВОДОЕМАХ

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Технологический процесс производства синтетических жирных кислот (СЖК) из нефтяных парафинов связан с образованием сточных вод, содержащих в своем составе пропионовую кислоту и ее натриевую соль. Гигиеническое нормирование содержания этих веществ в водоемах позволит обосновать требования к строительству очистных сооружений и контролировать эффективность их работы.

Пропионовая кислота CH_3CH_2COOH — легкоподвижная жидкость с резким запахом, она смешивается с водой в любых отношениях. Ее натриевая соль (пропионат натрия) — CH_3CH_2COONa — белый кристаллический порошок со слабым характерным запахом, хорошо растворимый в воде.

Пропионовая кислота и пропионат натрия придают воде ароматический запах и кисловато-вяжущий привкус. Порог ощущения запаха (интенсивность 1 балл) для пропионовой кислоты установлен на уровне 4,9 *мг/л*, практический порог (интенсивность 2 балла) — 11,5 *мг/л*, а для пропионата натрия — соответственно на уровне 13,7 и 28,9 *мг/л*. Нагревание водных растворов обоих соединений до 60° незначительно усиливает интенсивность запаха, кипячение же устраняет или значительно ослабляет его. Хлорирование воды, содержащей данные препараты, не вызывает провоцирования запаха. Пороговые концентрации пропионовой кислоты (порог восприятия и практический порог) по привкусу установ-

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лены на уровне 5,9 и 11,9 *мг/л*, а пропионата натрия — соответственно на уровне 9,9 и 21,4 *мг/л*.

Стабильность данных препаратов в воде определялась косвенными методами — по запаху и привкусу и по тесту на выживаемость дафний. Исследования показали, что пропионовая кислота и в еще большей степени пропионат натрия являются малостабильными соединениями.

Изучение влияния их на общий санитарный режим водоемов мы начали с опытов по биохимическому потреблению кислорода (БПК). Установлено, что пропионовая кислота в концентрациях до 0,1 *мг/л* и пропионат натрия в концентрациях до 0,3 *мг/л* практически не влияют на БПК. В пробах, содержащих указанные препараты от 0,1—0,3 до 15 *мг/л*, отмечается повышение БПК с первых же суток опыта. При этом обнаружена прямая зависимость усиления интенсивности процессов БПК от увеличения концентрации изучаемых веществ в воде. Так, через 5 суток инкубации БПК возрастает в пробах с 1 *мг/л* пропионовой кислоты или пропионата натрия на 17—20%, а в пробах с 5 *мг/л* уже на 83—96% по сравнению с контролем.

В настоящее время при гигиеническом нормировании веществ, повышающих уровень потребления кислорода, необходимо обязательно установить расход кислорода на весовую единицу вещества при завершении углеродистой фазы окисления, а также определить константу скорости окисления вещества (С. Н. Черкинский и С. Ф. Фридлянд). Судя по результатам исследований, при концентрациях пропионовой кислоты и ее натриевой соли, равных 1—5 *мг/л*, величина констант скорости потребления кислорода колеблется в пределах 0,1—0,17; это свидетельствует об окислении с той же скоростью, что и окисление бытовых сточных вод.

Время практического (на 99%) окончания процесса окисления данных веществ в воде мы устанавливали по формуле, выведенной В. М. Прусаковым. Из расчета на допустимую санитарным законодательством величину БПК полного 1 *мг* изучаемых веществ следует, что пороговыми являются концентрации пропионовой кислоты и пропионата натрия 2,5 *мг/л* для водоемов первого вида и 5 *мг/л* для водоемов второго вида. Однако эти концентрации увеличивают БПК соответственно на 40—50 и 80—90% по сравнению с контролем. В связи с этим для достижения большей санитарной надежности предельно допустимую концентрацию пропионовой кислоты и ее натриевой соли необходимо принять на уровне 1 *мг/л*, который вызывает увеличение БПК не более чем на 15—20%.

В литературе нет сведений о токсичности пропионовой кислоты для теплокровных животных. Что касается пропионата натрия, то описана только токсичность его технического образца (Хара Сабуро и соавт.). Нами изучалась токсикологическая характеристика пропионовой кислоты и ее натриевой соли в условиях острого, подострого и хронического санитарно-токсикологического эксперимента. Клиническая картина острого отравления указанными препаратами характеризовалась кратковременным периодом возбуждения, сменившимся длительным угнетенным состояния животного, одышкой, цианозом ушей и конечностей. Данные острого опыта, проведенного на белых мышах и белых крысах, обработаны статистическим методом пробит-анализа в модификации В. Б. Прозоровского. LD_{50} веществ для кроликов установлены по методу Дейхмана и Ле Бланка (см. таблицу).

Токсичность пропионовой кислоты и пропионата натрия для теплокровных животных в условиях острого опыта (в *г/кг*)

Вещество	Белые мыши	Белые крысы	Кролики
	$LD_{50} \pm m$		LD_{50}
Пропионовая кислота	1,37±0,15	1,51±0,25	1,9
Пропионат натрия . . .	7,8±0,6	8,15±0,91	6,75

Для суждения о кумулятивных свойствах препаратов был проведен подострый 20-дневный эксперимент, в течение которого белые крысы получали раздельно пропионовую кислоту и пропионат натрия из расчета соответственно $^1/_{10}$ и $^1/_5$ LD_{50}. На 21-й день опыта была введена доза веществ, равная среднесмертельной. Гибель животных наступила менее чем в 50% случаев.

В 2-месячном подостром эксперименте на фоне токсического действия указанных препаратов в дозе, равной $^1/_{20}$ LD_{50}, установлено, что наиболее существенным является снижение уровня сульфгидрильных (SH) групп в сыворотке и цельной крови животных. У белых крыс, получавших пропионовую кислоту в дозе, равной $^1/_{10}$ LD_{50}, отмечалось замедление прироста и снижение веса, уменьшение количества эритроцитов и содержания гемоглобина в крови, увеличение весового коэффициента печени. В хроническом санитарно-токсикологическом эксперименте на белых крысах-самцах в течение 6 месяцев испытывали дозы пропионовой кислоты 1, 10 и 50 *мг/кг* и пропионата натрия 5, 100 и 400 *мг/кг*. Исследовали изменение веса животных, содержание ретикулоцитов в крови, сульфгидрильных групп в сыворотке крови и состояния условнорефлекторной деятельности животных; внутренние органы животных были подвергнуты гистологическому исследованию.

Результаты исследований позволяют считать дозы пропионовой кислоты 1 *мг/кг* (20 *мг/кг*) и пропионата натрия 5 *мг/кг* (100 *мг/л*) недействующими по санитарно-токсикологическому признаку.

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Выводы

1. Пропионовая кислота и пропионат натрия способны ухудшать органолептические свойства воды, придавая ей ароматический запах и кисловато-вяжущий привкус. Пороговая концентрация пропионовой кислоты по влиянию на запах воды установлена на уровне 4,9 *мг/л*, а пропионата натрия по влиянию на привкус — на уровне 9,9 *мг/л*.

2. Наиболее существенным для пропионовой кислоты и ее натриевой соли является неблагоприятное влияние их на общий санитарный режим водоема и в первую очередь на процессы БПК. Пороговая концентрация каждого вещества по общесанитарному показателю установлена на уровне 1 *мг/л*.

3. Недействующей дозой пропионовой кислоты в условиях хронического токсикологического эксперимента следует считать 1 *мг/кг* (20 *мг/л*), пропионата натрия — на уровне 5 *мг/кг* (100 *мг/л*).

4. Лимитирующим признаком вредности для нормируемых веществ является общесанитарный. При определении условий спуска сточных вод, содержащих пропионовую кислоту и пропионат натрия, в открытые водоемы можно рекомендовать их предельно допустимые концентрации 1 *мг/л*.

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Effect in the Cow of Intraruminal Infusions of Volatile Fatty Acids and of Lactic Acid on the Secretion of the Component Fatty Acids of the Milk Fat and on the Composition of Blood

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1. The effects in the cow of intraruminal infusions of acetic acid, propionic acid or butyric acid on the secretion of the component fatty acids of the milk fat, and of these acids and of lactic acid on the composition of the blood plasma of the jugular vein, have been studied. 2. The infusion of acetic acid or butyric acid increased the yield of the C_4–C_{16} acids of milk fat but decreased the yield of C_{18} acids. The infusion of propionic acid decreased the yields of all major component acids except palmitic acid and possibly lauric acid. 3. The changes in the concentrations in blood plasma of glucose and of ketone bodies were consistent with the glucogenic effect of propionic acid and the ketogenic effects of butyric acid and acetic acid. The effects of lactic acid were not consistent from cow to cow. Only with the infusion of acetic acid was a significant increase in the concentration of total volatile fatty acids in blood plasma found. Infusions of butyric acid and of propionic acid tended to depress the concentration of citric acid in the blood plasma and infusion of acetic acid increased it. No consistent effects of the infused acids on the concentration in blood plasma of esterified cholesterol, free cholesterol, triglyceride or phospholipid were observed. 4. The possibility is discussed that the effects of the infused acids on milk-fat secretion are caused through an alteration of the concentrations of precursors of milk fat in mammary arterial blood.

Intraruminal infusions of volatile fatty acids in milking cows have been shown to have characteristic effects on milk-fat secretion; acetic acid and butyric acid increase the yield, whereas propionic acid decreases it (Rook & Balch, 1961; Rook, Balch & Johnson, 1965). To obtain information on the way in which these changes arise, the effects in lactating cows of intraruminal infusions of individual volatile fatty acids on the yields of the major fatty acids of milk fat and on the concentration in blood of some metabolites that are possibly of importance in milk-fat synthesis have been determined. In one experiment the effects of a continuous intraruminal infusion of lactic acid on blood composition also was determined. Preliminary findings on certain aspects of the work on blood composition have been published previously (Storry & Rook, 1961).

EXPERIMENTAL

Animals and their management. Lactating Friesian cows fitted with large permanent rumen fistulas were housed in an experimental byre in which the management routine was normal. The basal diet consisted of 14–16lb. of hay/day and a feed concentrate balanced for milk production given at a rate of 3–4lb./gallon of milk produced.

Experimental design. In the first series of experiments the effects of continuous intraruminal infusions of volatile fatty acids on the yield of the individual fatty acids of milk fat were determined. Acetic acid (1500ml./day), propionic acid (1500ml./day) and butyric acid (750ml./day) were each infused separately into two cows for periods of 1 week between initial and final control periods of the same length. Milk yield was recorded and samples of the milk were taken throughout the experiments. Weighted composite samples representing either a single day or, in certain experiments, during the initial control period, 2 days were prepared. Samples were analysed for fat content and the fatty acid composition of the milk fat.

In the second, third and fourth series of experiments the effects of intraruminal infusions of volatile fatty acids and of lactic acid on blood composition were determined, and in the third series the changes in blood composition were related also to the observed changes in milk-fat content. For the second series of experiments, infusions of propionic acid (1600ml./day) or butyric acid (1300ml./day) and of acetic acid (1500ml./day) or lactic acid (2000ml./day) were compared with infusions of water only, according to a Latin-square design, in successive experiments with groups of three cows. The treatment periods were of 4 weeks duration and, on the final day of each period, samples of blood from the jugular vein were taken through an indwelling polythene cannula at 0800, 1000, 1200, 1400, 1600 and 1800 hours.

In the third series of experiments, after a control period

of 1 week, a daily amount of 500ml. of butyric acid or of propionic acid was infused into the rumen and this was progressively increased until the animal began to refuse food. The addition of acid was then either stopped immediately or decreased for a day or two and then stopped. Milk yield was recorded and samples of milk were taken at each milking. Samples of blood from the jugular vein were taken through an indwelling polythene cannula at 0900, 1200 and 1500 hours on certain days during the control and experimental periods. Three experiments were done with propionic acid and five with butyric acid.

In the fourth series of experiments acetic acid, propionic acid and butyric acid were each infused separately in the same way as in the first series of experiments and blood samples were taken at 0900, 1200 and 1500 hours on each of two consecutive days during the period of acid infusion and also during the control periods.

Intraruminal infusion of the acids. The acids were of technical grade. Acetic acid, butyric acid and propionic acid were 96–97% pure and the main contaminants were other lower volatile fatty acids. The lactic acid (80%, w/w) contained about 72% of lactic acid and 8% of lactide. The acids were diluted with 50–60kg. of water and infused into the rumen by the technique of Rook & Balch (1961).

Preparation of blood samples. A 0·5% solution of citrate in 0·9% NaCl was injected into the indwelling polythene cannula as anticoagulant. When the citrate content of blood was to be determined, the citrate was replaced with heparin.

For analysis for lactic acid, pyruvic acid and α-oxoglutaric acid an ice-cold-trichloroacetic acid filtrate of whole blood [1vol. of blood to 5vol. of 10% (w/v) trichloroacetic acid solution] was prepared and then stored at −20°. For all other analyses, plasma was separated immediately and extracted for lipid analysis or stored at −20° until required.

Blood plasma lipids. To 225ml. of methanol (AnalaR grade; British Drug Houses Ltd., Poole, Dorset) in a glass-stoppered flask were added 30ml. of plasma (10ml. from each of three samples collected throughout a day) followed by 225ml. of chloroform (AnalaR grade; British Drug Houses Ltd.) and the mixture was placed in a water bath at 60° for 15min. The mixture was cooled to room temperature, a further 225ml. of chloroform was added, and then the mixture was shaken vigorously and filtered through a fast filter paper (Whatman no. 541). The filter paper was then washed with sufficient methanol–chloroform (1:2, v/v) to bring the total volume of the filtrate to 750ml. The lipid extract was purified by washing with 0·88% KCl (Folch, Lees & Sloane-Stanley, 1957) and dried to constant weight under a stream of nitrogen on a water bath at 50°.

The extracted lipids were separated on 3g. columns of silicic acid by the method of Barron & Hannahan (1958) as adapted by Moore & Doran (1962). The concentrations of the various major components of plasma lipid were calculated from the chemically determined cholesterol (Brown, 1959), glycerol (Moore, 1962) or phosphorus (Chen, Toribara & Warner, 1958) content of the appropriate fractions. The triglycerides and cholesterol esters were expressed as the oleate and the phospholipid concentration was calculated by multiplying the determined P by 25. In this way contamination of fractions with other compounds and with silicic acid was avoided (Storry & Rook, 1964*a*).

The cholesterol esters, free cholesterol, triglycerides and phospholipids, determined as described, accounted for about 95% of the total lipid applied to the column.

Methods of analysis for other plasma constituents. The total steam-volatile fatty acids of plasma were determined by steam-distillation (Searisbrick, 1952) and the acids in the distillate were separated by gas–liquid chromatography (Storry & Millard, 1965). Details of other methods of analysis are as follows: ketone bodies (Reid, 1960), glucose (Somogyi, 1952), citric acid (McArdle, 1955), lactic acid (Barker & Summerson, 1941; Pennington & Sutherland, 1956) and pyruvic acid and α-oxoglutaric acid (Friedman & Haugen, 1943).

Extraction of milk lipids and determination of fatty acids. The fat from milk samples was extracted by the technique of Folch *et al.* (1957) (Storry & Millard, 1965) and stored in methanol–chloroform (1:2, v/v) under nitrogen until required for analysis. Then 150ml. of the lipid solution was evaporated to dryness and the lipid hydrolysed with 10% (w/v) KOH in methanol–water (1:1, v/v) under reflux for 3hr. at 100°. The acids were recovered in light petroleum (b.p. 40–60°) and the methyl esters prepared by using methanol–boron trifluoride in H_2SO_4 (Metcalfe & Schmitz, 1961). The esters were analysed for C_{12}–C_{20} acids by gas–liquid chromatography by using a Pye argon chromatograph (W. G. Pye and Co. Ltd., Cambridge). The relative proportions of the esters were determined from the retention times and peak heights by the method of Carroll (1961). Peaks were identified by comparison with authentic esters.

The remaining lipid was hydrolysed and the lower fatty acids were separated from the acidified solution by steam-distillation and analysed by gas–liquid chromatography (Storry & Millard, 1965). The residual higher fatty acids were recovered and weighed.

RESULTS

Effects of infusions on the yields of milk fat and of individual acids in milk fat

The changes in the yield of milk fat observed in response to the intraruminal infusions of individual volatile fatty acids were consistent with previous results (Rook & Balch, 1961; Rook *et al.* 1965). The mean changes ± S.E.M. in fat yield, expressed as percentages of the original daily yield for each of the two cows, were +8·8±3·1 and +6·7±2·6 respectively with the infusion of acetic acid, −12·1±7·5 and −13·4±3·5 with the infusion of propionic acid and +9·3±3·7 and +10·1±5·7 with the infusion of butyric acid. The associated changes in the yields of the major component fatty acids of milk fat are given in Table 1. The patterns of response were the same in both cows. The infusions of acetic acid and of butyric acid had similar effects: the yields of C_4–C_{16} acids were invariably increased, and to a similar extent, whereas, with the exception of the yield of $C_{18:2}$ acid plus $C_{18:3}$ acid in cow 1, the yields of all the C_{18} acids were invariably depressed. During the infusion of propionic acid the yield of palmitic acid was unaltered, but the yields of all other major fatty acids, with the possible exception of lauric acid, were decreased.

Table 1. *Effects in lactating cows of intraruminal infusions of acetic acid, of propionic acid and of butyric acid on the yields of milk fat and of the major individual fatty acids of milk fat*

Experimental details are given in the text. The designation of the fatty acids is according to the nomenclature of Dole, James, Webb, Rizack & Sturman (1959).

	Cow 1		Cow 2	
	Mean yield during control periods (g./day)	Percentage change with infusion of acid	Mean yield during control periods (g./day)	Percentage change with infusion of acid
Acetic acid infusion				
Milk fat	526	+9	558	+13
Fatty acids				
4:0	12·5	+22	14·5	+5
6:0	8·6	+28	9·7	+11
8:0	5·3	+27	6·2	+17
10:0	9·9	+30	13·0	+16
12:0	16·5	+5	16·3	+26
14:0	6·2	+14	60·9	+28
16:0	149·4	+25	141·9	+38
18:0	36·7	−20	42·5	−20
18:1 *cis*	109·6	−20	101·4	−21
18:1 *trans*	14·3	−13	14·1	−10
18:2, 18:3	11·7	+10	11·7	−14
Propionic acid infusion				
Milk fat	770	−13	753	−12
Fatty acids				
4:0	26·0	−49	35·3	−24
6:0	22·3	−48	25·9	−30
8:0	14·7	−45	16·0	−33
10:0	24·9	−80	27·0	−16
12:0	31·2	−20	26·0	+6
14:0	86·5	−11	76·6	−15
16:0	209·0	+1	228·0	−2
18:0	72·0	−47	51·0	−37
18:1 *cis*	138·0	−27	144·0	−28
18:1 *trans*	22·4	−18	29·0	−24
18:2, 18:3	17·1	−11	17·7	−41
Butyric acid infusion				
Milk fat	691	+7	951	+3
Fatty acids				
4:0	19·3	+8	25·2	+48
6:0	14·4	+20	18·3	+54
8:0	9·4	+25	10·7	+56
10:0	15·3	+31	17·7	+13
12:0	22·6	+30	24·0	+39
14:0	64·4	+30	78·6	+39
16:0	183·0	+15	207·5	+28
18:0	49·0	−6	61·5	−6
18:1 *cis*	130·0	−31	171·0	−20
18:1 *trans*	24·4	−11	25·1	−14
18:2, 18:3	17·8	−24	18·3	−12

Effects of intraruminal infusions on non-lipid constituents of blood

Glucose and ketone bodies (*Table* 2). The infusion of butyric acid invariably caused an increase in the concentration of ketone bodies and a decrease in the concentration of glucose in the blood plasma, and acetic acid had a similar though smaller and less consistent effect. Conversely, propionic acid invariably increased the concentration of glucose and decreased that of ketone bodies, and lactic acid had no consistent effect. These effects of acetic acid,

Table 2. *Effects of intraruminal infusions of propionic acid, of butyric acid, of acetic acid and of lactic acid on the concentrations of non-lipid components in the plasma*

Experimental details are given in the text. The values represent the mean concentrations ± S.E.M. throughout 12hr. periods. *$P=0{\cdot}05$, **$P=0{\cdot}01$ and ***$P=0{\cdot}001$, where P is the probability that the observed difference could have arisen by chance.

Blood constituent	Cow	Water infusion	Propionic acid infusion	Butyric acid infusion	Water infusion	Acetic acid infusion	Lactic acid infusion
Acetone + acetoacetic acid (expressed as mg. of acetone/100 ml.)	4	0·16 ± 0·045	0·15 ± 0·031	3·90 ± 0·323***	0·14 ± 0·050	0·40 ± 0·040**	0·15 ± 0·036
	5	—	—	—	0·16 ± 0·045	—	0·16 ± 0·017
	6	0·12 ± 0·017	0·07 ± 0·020	3·34 ± 0·384***	—	—	—
	7	0·37 ± 0·063	0·15 ± 0·017**	5·25 ± 0·329***	0·26 ± 0·038	0·99 ± 0·115***	0·26 ± 0·030
β-Hydroxybutyric acid (mg./100 ml.)	4	5·42 ± 0·536	3·12 ± 0·466*	77·69 ± 5·53***	4·79 ± 0·472	14·28 ± 2·415**	3·10 ± 0·380*
	5	—	—	—	5·37 ± 0·653	—	7·77 ± 0·739*
	6	6·52 ± 0·671	2·52 ± 0·415***	65·87 ± 6·51***	—	—	—
	7	8·70 ± 1·747	3·48 ± 0·326*	109·24 ± 6·15***	8·80 ± 0·820	19·98 ± 2·463**	9·26 ± 0·619
Glucose (mg./100 ml.)	4	56·8 ± 6·17	65·0 ± 5·07	40·1 ± 6·33	65·9 ± 1·58	66·2 ± 2·16	63·3 ± 1·70
	5	—	—	—	57·7 ± 0·97	—	63·0 ± 3·26
	6	63·9 ± 1·62	74·6 ± 2·69**	48·4 ± 3·30**	—	—	—
	7	56·0 ± 2·79	73·9 ± 2·22***	28·1 ± 3·14**	65·6 ± 1·85	51·3 ± 3·27*	72·8 ± 1·53*
Citric acid (mg./100 ml.)	4	4·88 ± 0·632	3·52 ± 0·212	2·62 ± 0·764*	4·56 ± 0·127	6·04 ± 0·293***	4·66 ± 0·084
	5	—	—	—	3·44 ± 0·114	—	1·88 ± 0·071***
	6	2·48 ± 0·737	—	2·90 ± 0·583	—	—	—
	7	5·08 ± 0·114	4·60 ± 0·055**	3·96 ± 0·130***	4·42 ± 0·089	5·10 ± 0·272*	5·22 ± 0·114***
Lactic acid (mg./100 ml.)	4	2·99 ± 0·145	7·00 ± 0·327***	4·07 ± 0·134*	2·68 ± 0·145	3·91 ± 0·430*	3·31 ± 0·399
	7	4·03 ± 0·232	5·02 ± 0·152**	3·50 ± 0·127	3·84 ± 0·232	3·91 ± 0·367	3·00 ± 0·255*
Total volatile fatty acids (m-equiv./l.)	4	0·57 ± 0·032	0·50 ± 0·055	0·53 ± 0·063	0·64 ± 0·030	4·10 ± 0·937***	0·46 ± 0·020***
	5	—	—	—	0·53 ± 0·055	—	0·55 ± 0·032
	6	0·50 ± 0·088	0·39 ± 0·230	0·71 ± 0·120	—	—	—
	7	0·87 ± 0·101	0·58 ± 0·103	0·99 ± 0·161	1·06 ± 0·093	3·54 ± 0·379***	0·88 ± 0·057
Acetic acid† (m-equiv./l.)	4	0·53	0·47	0·44	0·61	4·02	0·42
	5	—	—	—	0·49	—	0·50
	6	0·47	0·33	0·64	—	—	—
	7	0·83	0·54	0·89	1·01	3·46	0·81
Propionic acid† (m-equiv./l.)	4	0·02	0·02	0·04	0·02	0·05	0·02
	5	—	—	—	0·02	—	0·03
	6	0·01	0·05	0·02	—	—	—
	7	0·02	0·03	0·03	0·03	0·04	0·03
Butyric acid† (m-equiv./l.)	4	0·02	0·04	0·02	0·02	0·03	0·02
	5	—	—	—	0·02	—	0·03
	6	0·01	0·01	0·06	—	—	—
	7	0·02	0·01	0·07	0·02	0·04	0·03

† Distillates from determinations of total volatile fatty acids were combined on a daily basis and used for the determination of individual acids.

propionic acid and butyric acid in lactating cows are similar to those obtained in short-term experiments with starved sheep (Annison, Hill & Lewis, 1957; Armstrong & Blaxter, 1957; Clark & Malan, 1956), goats (Schultz & Smith, 1951; Johnson, 1955) and cows (Waldo & Schultz, 1960). They are also in line with the conclusions of Annison, Leng, Lindsay & White (1963) that, when labelled acids were infused into the portal vein of sheep, whereas propionate is glucogenic, butyrate and, to a less extent, acetate are ketogenic in their metabolism. The variable results obtained with lactic acid were probably related to the fact that in the rumen this acid is converted into a mixture of propionic acid and butyric acid (Hueter, Shaw & Doetsch, 1956) and the composition of the mixture may vary from cow to cow.

Volatile fatty acids (*Table* 2). Acetic acid was the only acid infused that appeared itself in the plasma and caused a marked increase in the concentration of total volatile fatty acids. Propionic acid or butyric acid caused only small increases in their respective concentrations in the plasma, indicating that the added acids were almost completely metabolized in the rumen epithelium or in the liver. Propionic acid, however, depressed acetate concentration in the plasma, as found by Annison *et al.* (1963). In two cows, lactic acid also produced the same effect.

Citric acid (*Table* 2). The infusion of butyric acid caused a marked fall in the concentration of citric acid in blood plasma, and propionic acid tended to have a similar effect although the response was more variable. In contrast, acetic acid tended to increase the plasma citric acid concentration. Lactic acid had no consistent effect.

Lactic acid (*Table* 2). The lactic acid concentration in the blood plasma was increased consistently only by the infusion of propionic acid. Lactic acid production from propionic acid by sheep-liver slices has been demonstrated *in vitro* (Leng & Annison, 1963).

Keto acids. In no instance did the intraruminal infusion of the volatile fatty acids produce a measurable change in the concentration of plasma keto acids.

The third series of experiments confirmed fully the effects on blood composition observed in the second series, and detailed results are therefore not reported. One important point that did emerge, however, was the variable degree to which animals responded to the infusion of acids, in terms both of the effects on blood composition and on fat yield. This is illustrated in Figs. 1 and 2 with results obtained with two cows, 8 and 9, giving a similar milk yield and maintained on a similar diet. Changes in β-hydroxybutyrate and glucose concentrations in blood plasma were much more marked in cow 9 with the infusion both of propionic acid and of

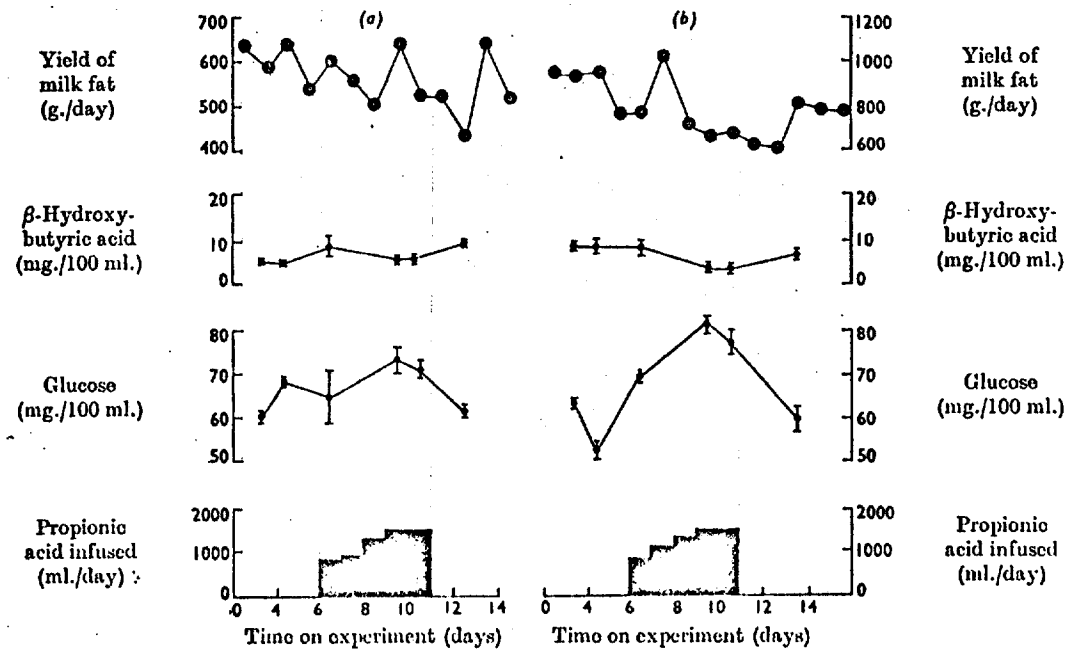

Fig. 1. Effect of intraruminal infusions of propionic acid on the yield of milk fat and on the concentrations of β-hydroxybutyric acid and glucose in the blood plasma. Experimental details are given in the text. (*a*) Cow 8; (*b*) cow 9.

butyric acid. The daily yield of fat, however, was unaltered in cow 9 by the infusion of butyric acid (mean percentage change, $+1{\cdot}2 \pm 8{\cdot}8$) whereas it tended to be depressed with the infusion of propionic acid (mean percentage change, $-9{\cdot}6 \pm 7{\cdot}1$), but with cow 8 the infusion of butyric acid markedly increased the fat yield (mean percentage change, $+13{\cdot}4 \pm 3{\cdot}8$) and the infusion of propionic acid was without effect

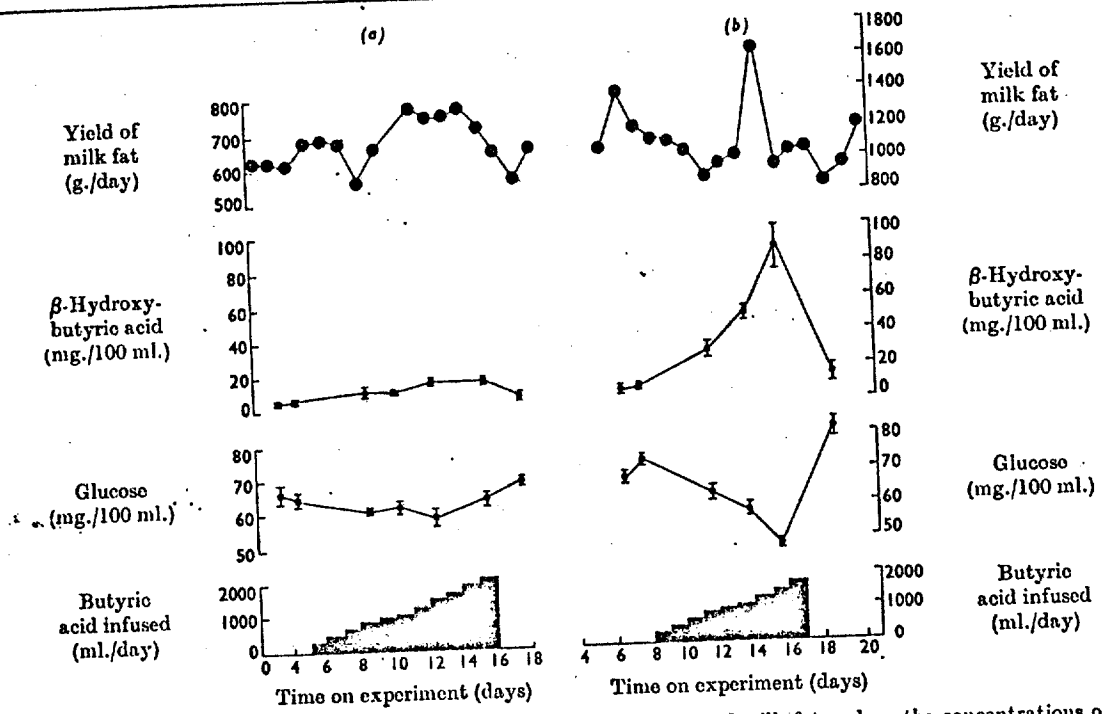

Fig. 2. Effect of intraruminal infusions of butyric acid on the yield of milk fat and on the concentrations of β-hydroxybutyric acid and glucose in the plasma. Experimental details are given in the text. (*a*) Cow 8; (*b*) cow 9.

Table 3. *Effects of intraruminal infusions of acetic acid, of propionic acid and of butyric acid on the concentrations of the four major fractions of lipids in the plasma of lactating cows*

Experimental details are given in the text. Values are given as the means ± S.E.M.

Cow	Infusion given	Cholesterol esters (mg./100 ml.)	Free cholesterol (mg./100 ml.)	Triglycerides (mg./100 ml.)	Phospholipid (mg./100 ml.)
1	Water only	170 ± 38·4	23·3 ± 5·43	4·3 ± 1·50	117 ± 34·1
	Butyric acid	158 ± 15·1	22·7 ± 3·64	3·7 ± 1·31	85 ± 8·0
2	Water only	178 ± 19·8	25·3 ± 2·60	6·4 ± 2·02	116 ± 11·9
	Butyric acid	194 ± 29·6	28·5 ± 1·81	7·4 ± 2·35	121 ± 6·7
3	Water only	178 ± 21·5	27·1 ± 1·27	7·1 ± 0·57	126 ± 9·0
	Butyric acid	174 ± 24·7	29·1 ± 1·35	3·6 ± 0·71	127 ± 1·4
1	Water only	151 ± 3·4	24·1 ± 1·01	5·6 ± 1·07	98 ± 11·2
	Propionic acid	131 ± 18·3	29·6 ± 0·57	4·4 ± 0·38	95 ± 25·4
2	Water only	192 ± 45·1	29·6 ± 0·50	4·4 ± 0·17	158 ± 1·6
	Propionic acid	190 ± 4·9	26·8 ± 2·93	4·6 ± 0·95	139 ± 0·8
3	Water only	173 ± 57·1	24·4 ± 1·54	2·7 ± 1·05	122 ± 2·1
	Propionic acid	159 ± 10·7	28·5 ± 2·45	2·4 ± 0·22	106 ± 1·8
1	Water only	151 ± 3·4	24·1 ± 1·01	5·6 ± 1·07	99 ± 11·2
	Acetic acid	152 ± 3·5	22·8 ± 1·00	5·5 ± 0·85	100 ± 2·1
2	Water only	192 ± 45·0	29·6 ± 0·50	4·4 ± 0·17	158 ± 1·5
	Acetic acid	197 ± 3·3	26·1 ± 1·1	4·2 ± 0·22	120 ± 6·8

(mean percentage change, $-0{\cdot}4 \pm 6{\cdot}3$). The observed changes in total fat yield are thus not closely dependent on associated changes in the concentrations of β-hydroxybutyrate or of glucose in blood plasma. Because of the large day-to-day variations in fat yield (a recognized problem inherent in the use of routine milking techniques) a statistical study of the relationship between milk-fat secretion and the concentrations of individual constituents in blood plasma was not practicable.

Effects of infusions on lipid constituents of blood plasma

No consistent effects of infusions of volatile fatty acids on the concentrations in the plasma of esterified or free cholesterol, triglycerides or phospholipids were observed (Table 3). There was considerable day-to-day variation in the concentrations of particularly the esterified cholesterol and phospholipid fractions.

DISCUSSION

The increases in the yield of milk fat that occur in the lactating cow in response to the intraruminal infusion of acetic acid or of butyric acid must result primarily from an increased synthesis and secretion of C_4–C_{16} fatty acids. The associated decrease in the secretion of C_{18} acids is probably a compensatory change, which would result from an increase in the amount of C_4–C_{16} acids in a common pool of fatty acids contributing to triglyceride formation in the mammary gland. The decrease in the yield of milk fat in response to the intraruminal infusion of propionic acid does not, however, result from simply the opposite of these changes: the secretion of palmitic acid is not affected and the secretion of all other major constituent acids, with the possible exception of lauric acid, is decreased.

Responses in milk-fat secretion to intraruminal infusions of volatile fatty acids are at a maximum within about 4 days of the introduction of an infusion, and by comparison with corresponding changes in the secretion of milk protein or in the yield of milk (Rook & Balch, 1961) are rapid in development. It is therefore possible that the rate of synthesis of milk fat is sensitive to short-term variations in the concentration of its precursors in arterial blood. Because of technical difficulties, serial samples of mammary arterial blood cannot readily be obtained from milking cows, and in the present work it was assumed that variations in the concentrations of materials in arterial blood would be reflected in the composition of jugular venous blood, which can be readily sampled.

In a preliminary account (Storry & Rook, 1961) of some of the present results, the suggestion was made that the decrease and the increase in the yield of milk fat brought about respectively by the infusion of propionic acid and of butyric acid might be related to the associated changes in the concentration of β-hydroxybutyrate in blood plasma, since β-hydroxybutyrate is a possible precursor of the short-chain fatty acids of milk fat (Kumar, Lakshmanan & Shaw, 1959). Similarly, the increase in milk-fat yield in response to the infusion of acetic acid was attributed partly to the associated increase in the concentration of β-hydroxybutyrate in blood plasma but also, because of the known role of acetate in milk-fat synthesis (Cowie *et al.* 1951; Popják, French, Hunter & Martin, 1951; Rogers & Kleiber, 1957; Hardwick, Linzell & Mepham, 1963), to the associated increase in the concentration of the latter in blood plasma.

That changes in the secretion of the fatty acids of milk fat with intraruminal infusions of propionic acid are not the opposite of those obtained with the infusions of butyric acid now excludes the possibility that the effects of these two acids can be entirely accounted for by an altered synthesis of fatty acids in response to a change in the concentration of precursor β-hydroxybutyrate. Of particular interest therefore is the observation by Vallance & McClymont (1959) that intravenous infusions of glucose in a lactating cow depressed milk-fat secretion, and their suggestion that, since there was a concomitant depression in the concentration of plasma triglycerides and a decrease in the mammary arteriovenous difference (McClymont & Vallance, 1962), the effect may have been due to a decreased synthesis of milk fat from triglycerides of the blood plasma. In support of this possibility there is now good evidence for the formation of more particularly the long-chain acids of milk fat from triglycerides of the chylomicra and the low-density lipoprotein of the blood plasma (Barry, Bartley, Linzell & Robinson, 1963; Welch, Noble, Wright, Bishop & Glascock, 1963; Lascelles, Hardwick, Linzell & Mepham, 1964), and also the intravenous infusion of an emulsion of cotton-seed oil (Storry & Rook, 1964*b*) has been shown to increase the secretion of milk fat.

The intraruminal infusion of propionic acid would presumably exert an effect similar to the intravenous infusion of glucose, since propionic acid is glucogenic in its metabolism and its infusion in lactating cows has invariably caused an increase in plasma glucose concentration. However, in the present experiments no depression in the concentration of plasma triglycerides was observed in response to the intraruminal infusion of propionic acid, but this may be due to the fact that in the cows examined the recorded concentrations of triglycerides were low and variable. Also, the changes in blood glucose concentration and in fat yield in response to the

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infusion of propionic acid were much smaller than those obtained by McClymont & Vallance (1962) during the intravenous infusion of glucose. Smaller changes in plasma triglyceride concentration in response to the infusion of propionic acid might therefore be expected, and these would be difficult to detect. If it is assumed that the decrease in the secretion of C_{18} acids in response to the intraruminal infusion of propionic acid or to the intravenous infusion of glucose results from a diminished synthesis of these acids from plasma triglycerides, any decrease in the secretion of the short-chain fatty acids of milk fat could then reasonably be attributed to a decrease in the concentration of plasma β-hydroxybutyrate. The evidence of Kumar *et al.* (1959) is that β-hydroxybutyrate is incorporated into mainly the C_4–C_{10} fatty acids of milk fat. Some explanation, however, is then required for the marked increase in the yield of palmitic acid obtained in response to the intraruminal infusion of butyric acid. One possibility would be that the metabolism of butyrate in the liver gives rise to acetate (Annison *et al.* 1963) and thus promotes the incorporation of acetate into milk fat. But in the present experiments a net conversion of butyrate into acetate in the liver was not indicated, since the concentration of acetate in peripheral blood was unaltered by the intraruminal infusion of butyric acid, and Rook *et al.* (1965) have already shown that intraruminal infusions of butyric acid have a more marked effect on fat yield than do infusions of acetic acid, and that when the infusions are given together the effects are additive.

The increased secretion of the C_4–C_{16} acids observed with the intraruminal infusion of acetic acid is consistent with the hypothesis suggested previously (Storry & Rook, 1961) of an increased synthesis of fatty acid from acetate due to the increased concentration of acetate in peripheral blood.

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Identification of Acetic, Propionic, and Sorbic Acids in Bakery Products by Thin Layer Chromatography

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A rapid and sensitive thin layer chromatographic method has been described for detecting acetic, propionic, and sorbic acids in bakery products. Acetic, propionic, and sorbic acids were steam distilled from an acidified mixture of the samples into a solution of NaOH. After concentration of the alkaline solutions, samples were spotted on a thin layer plate; the chromatoplate was developed and the acids were identified by spraying with bromcresol purple.

Propionic acid, calcium propionate, and sorbic acid are commonly used as mold inhibitors and preservatives. Several paper chromatographic procedures for the detection of the acids have been reported (1, 2). The official final action AOAC method for the identification of acetic and propionic acids is a paper chromatographic procedure in which spraying with methyl red-bromothymol blue and exposure to ammonia vapors are used for detection (3). Many authors have also reported thin layer chromatographic techniques for the separation and identification of preservatives (4–9). However, these procedures are not as sensitive as desired. In addition, because of the volatility of propionic acid, especially, the spots used for identification fade rapidly.

We have developed a thin layer chromatographic method for the separation of acetic, propionic, and sorbic acids in bakery products; it is sensitive and yields relatively stable chromatographic spots. The sample, bakery products in this study, is steam distilled and the sodium salts of the preservatives are prepared and spotted on the TLC plates. The method given below is applicable to all leavened bakery products, including non-yeast leavened products.

METHOD

Reagents

(a) *Mobile solvents.*—(*1*) *n*-Butanol-25% NH_4OH-water (7 + 1 + 2). Prepare fresh daily; use organic layer. (*2*) *n*-Propanol-25% NH_4OH-ethanol (7 + 1 + 2).

(b) *Chromogenic reagent.*—0.04% Bromcresol purple in 50% ethanol. Adjust to pH 10 with 0.1*N* NaOH.

(c) *Standard solution.*—Dissolve in ethanol 250 mg each of acetic and propionic acids and 50 mg sorbic acid in separate 100 ml volumetric flasks. Bring to volume with ethanol.

(d) *Ethanol.*—96%.

Apparatus

(a) *Steam distillation apparatus.*—(Scientific Glass Apparatus Co., Bloomfield, N.J. 07003) consists of: (*1*) *Steam generator.*—Gas-fired; (*2*) *Distillation flask.*—500 ml round-bottom boiling flask with ₮ B 24 joint; (*3*) *Splash head.*—With pear bulb, vertical delivery tube, and male connections ₮ B 24 at bottom and side; (*4*) *Liebig condenser.*—With female connections ₮ B 24 at top and male connections ₮ B 24 at bottom; and (*5*) *Receiver adapter.*—With female connection ₮ B 24 at top.

(b) *Rotary evaporator.*—Büchi Rotavapor (W. Büchi Glasapparatefabrik, Flawil, Switzerland) or equivalent.

(c) *TLC apparatus.*—Glass plates 20 × 20 cm, Desaga applicator, mounting board, spotting template, 10 µl microsyringe, desiccating storage cabinet, storage rack, glass developing chamber, and sprayer.

(d) *Heating oven.*—100°C.

Sample Preparation

Weigh 20 g air-dried bakery product in 500 ml round-bottom distillation flask, add 100 ml water and 10 ml 4*N* H_3PO_4, and mix. Mixture should now be acid to congo red paper.

Put flask head on distillation flask and connect to condenser with receiver adapter and to steam generator. Heat distillation flask and distill 200 ml in ½ hr into flask containing 50 ml water and 5 ml 0.1*N* NaOH. Concentrate this solution to ca 10 ml in rotary evaporator (ca 45 min).

Detection

Spot two 5 µl samples with gentle air draft on prepared plate of 0.25 mm silica gel G (activated); also spot 5 µl standard solutions. Place plate in chromatographic chamber and develop chromatoplate ca 12 cm (ca 2 hr). Remove plate and dry 5 min in oven at 100°C. Let plate cool to room temperature. Spray plate with chromogenic reagent and place in oven at 100°C. Remove plate after 5 min; acids will then

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be seen as yellow spots on light blue background which turns yellow after standing because of hydrolysis of the ammonium salts in the silica gel.

Results and Discussion

The technique described in this paper is simple, rapid, and very sensitive. The alkali salts of the preservatives rather than the free acids are spotted on the plate. This is a great advantage in the case of acetic and propionic acids which are volatile.

A mobile phase which contains ammonia is used in order to form the ammonium salts of the acids and the silica gel of the plate. When heated 5 min in an oven at 100°C, the ammonium salts decompose, resulting in the formation of the free acids; the spots of these acids are more acidic than the silica gel layer of the plate. After spraying with bromcresol purple solution, the acids appear as yellow spots on a light blue background. Immediately after spraying, the ammonium salts of the silica gel are not completely hydrolyzed; on standing, hydrolysis is completed and the background turns yellow. The acids are then seen as yellow spots on a light yellow background. The color is stable for at least 8 hr with no perceptible change occurring during this period.

Table 1. R_f values of acetic, propionic, and sorbic acids in 2 solvent systems[a]

Acid	R_f Solvent 1	R_f Solvent 2
Acetic	0.13	0.36
Propionic	0.16	0.42
Sorbic	0.27	0.51

[a] Solvent 1: *n*-butanol-25% NH_4OH-water (7 + 1 + 2); solvent 2: *n*-propanol-25% NH_4OH-ethanol (7 + 1 + 2).

R_f values for the 3 acids in 2 solvent systems are shown in Table 1. The R_f values of acetic and propionic acids are close but the identification of both acids in a sample is quite easy, especially in solvent system 2 (*see* Table 1). The study to find another solvent system for better resolution will be continued.

About 10 μg acetic and propionic acids and about 2.5 μg sorbic acid can be detected. Although sensitivity was not the main objective of this report, the method is more sensitive than some of those in the current literature. In addition, this method is applicable to all leavened bakery products, including non-yeast leavened products.

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obsolete, nor that it replaces other in vitro tests for certain purposes. It is suggested, however, that real application will be found in laboratories where it is desired to assay insulin to a high level of precision by a biological method but without an extensive outlay in animals and facilities. A further advantage is that only small quantities of insulin, in the region of 0.1 unit, are required, which is particularly useful if the material is scarce.

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STUDIES ON THE IN VITRO ANTITUMOR ACTIVITY OF FATTY ACIDS

III. SATURATED MONOCARBOXYLIC ACIDS[1]

Susan Tolnai and Joseph F. Morgan

Abstract

Previous studies on the in vitro antitumor activity of 10-hydroxy-2-decenoic acid from royal jelly and of saturated dibasic fatty acids have been extended to a series of saturated monocarboxylic acids ranging from C_3 to C_{18}. A widespread ability to inhibit the development of the ascites forms of the 6C3HED lymphosarcoma, Ehrlich carcinoma, and TA_3 mammary carcinoma has been found under acid pH conditions. In general, the antitumor activity of the saturated monocarboxylic acids was most pronounced in compounds with a carbon chain length of C_8 to C_{11}. One member of this series, capric (decanoic) acid, was found to possess marked antitumor activity at physiological pH.

Introduction

Previous studies from this laboratory have shown (1, 2) that royal jelly, when mixed with tumor cells prior to inoculation of mice, completely suppressed the formation of three lines of ascites tumors and the development of transplantable AKR leukemia. Fractionation studies (2) established that the antitumor activity resided mostly, if not entirely, in 10-hydroxy-2-decenoic acid, the main fatty acid component of royal jelly. Under strictly defined conditions, and at pH values below 4.5, tumor-inhibiting activity was demonstrated with saturated dicarboxylic acids in the series of chain length from C_3 to C_{10} (3). In an effort to elucidate the possible relationship between the chemical structure of fatty acids and their antitumor activity, these investigations were extended to other types of fatty acids. The present communication reports the results obtained when monocarboxylic acids of chain length from C_3 to C_{18} were tested for antitumor activity against three lines of ascites tumors in mice.

Materials and Methods

In vitro antitumor studies were carried out with three lines of ascites tumors: the 6C3HED lymphosarcoma, the Ehrlich ascites carcinoma, and the TA_3 mammary carcinoma. The 6C3HED lymphosarcoma was carried by weekly intraperitoneal passage in C3H mice. The other two ascites tumors were also carried by weekly serial passages, but in the non-specific Connaught mouse strain (4). All antitumor experiments were carried out in this non-specific mouse strain, using male mice, 20 to 22 g in weight. The C3H mice used in these experiments were purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and the Connaught mice from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

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The ascitic material was harvested by intraperitoneal puncture 6 to 8 days after inoculation, mixed with two volumes of deionized water to lyze any erythrocytes present and the tumor cells collected by centrifugation at 1500 r.p.m. for 3 minutes. The packed cells were resuspended in sufficient Hanks' balanced salt solution (5) to yield a suspension containing 10 to 15 million cells per milliliter, as determined by direct cell count in a Spencer-Neubauer hemocytometer.

The monocarboxylic acids to be tested were incorporated in graded amounts in Hanks' solution, the washed cell suspension added, and the pH of the reaction mixture adjusted to the desired level with 0.1 *N* HCl or 0.1 *N* NaOH, using a Beckman pH meter. Complete details of these methods have been published previously (2).

When fatty acids containing 10 or more carbon atoms were to be tested, it was found necessary to use Tween 80 (obtained from the Atlas Powder Co., Wilmington, Delaware) in a 2% weight per volume concentration as an emulsifying agent. The Tween 80 was added to the Hanks' solution diluent used in preparing cell suspensions for the inoculation of both test and control mice and at this concentration was found to have no effect upon the survival time.

Groups of 10 mice were injected intraperitoneally with 0.5 ml per mouse of the reaction mixtures as rapidly as possible, and, at the same time, groups of control mice received the same number of tumor cells without admixture of the fatty acids. It was found that any deaths caused specifically by tumors occurred within the first 60 days following inoculation. Accordingly, all test mice were kept under daily observation for an arbitrary period of 90 days, at which time the experimental results were recorded. However, weekly observation of the mice was continued for a total period of 1 year, and at the end of this time all surviving animals were autopsied and examined for possible tumor growth.

The monocarboxylic acids used in these experiments were of the highest quality available commercially, and were used without further purification.

Results

In Vitro Antitumor Activity of Monocarboxylic Acids

In Table I are summarized the results of experiments carried out with Ehrlich ascites cells mixed with varying amounts of monocarboxylic acids ranging in chain length from C_3 to C_{18}. In all cases, the pH of the reaction mixtures was kept below 4.5. With the exception of butyric and valeric acids, all the fatty acids in this series caused complete protection against the inoculated tumor cells. Similar results were obtained when 6C3HED or TA_3 ascites tumor cells were used, but some minor differences in the responsiveness of the individual tumor types were observed.

Relationship between Concentration and Activity of Monocarboxylic Acids

In order to determine the relationship between concentration and activity of the monocarboxylic acids, each tumor cell type was tested over a wide range of

TABLE I

Effect of monocarboxylic acids when mixed with Ehrlich ascites carcinoma cells at pH 4.5 prior to inoculation of mice*

Fatty acid		Concentration (mg per ml)	Average survival (days) Test	Average survival (days) Control	Survivors (90 days)
Propionic	(C_3)	1.6	27†	11	2/10
		3.2	∞‡	12	9/9
Butyric	(C_4)	3.2	54	12	7/10
Valeric	(C_5)	3.2	28	10	8/10
Caproic	(C_6)	1.6	39	14	9/10
		5.0	∞	14	10/10
Caprylic	(C_8)	1.6	42	13	8/10
		2.5	∞	13	10/10
Capric (decanoic)	(C_{10})	1.0	20	13	9/10
		2.0	∞	13	10/10
Lauric	(C_{12})	1.0	22	15	8/10
		2.0	∞	15	10/10
Myristic	(C_{14})	0.5	9	11	0/10
		1.0	∞	17	10/10
Palmitic	(C_{16})	0.5	8	11	0/10
		1.0	∞	17	10/10
Stearic	(C_{18})	1.0	27	17	0/10
		2.0	∞	15	10/10

*Each mouse received 5 to 8 million tumor cells either alone (control) or mixed with monocarboxylic acid (test).
†Calculated from the death times of the mice which failed to survive.
‡Represents survival beyond the 90-day test period.

fatty acid concentrations under acid pH conditions. Representative data are summarized in Table II. The amount of fatty acid required for complete suppression of tumor growth in most cases was only slightly higher than the partially protective or ineffective doses. However, the range between effective and ineffective concentrations was generally wider than that found with most dicarboxylic acids tested previously (3).

Variations in Tumor Susceptibility

Variations in susceptibility to the monocarboxylic acids were found among the three tumors, as summarized in Table III. Only two of the fatty acids tested, capric and myristic acids, protected the inoculated mice against all three ascites tumors at the same concentration. In general, the 6C3HED lymphosarcoma proved to be the most sensitive of the three tumors used and produced results similar to those obtained previously with the dicarboxylic acid series (3).

The minimal effective concentration, i.e., the smallest amount of fatty acid which gave total protection in all mice inoculated, could not be determined exactly with palmitic and stearic acids against the 6C3HED lymphosarcoma and the TA_3 mammary carcinoma. In these cases, the highest levels of the fatty acids tested (2.0 mg per ml) gave only partial protection, but since emulsi-

TABLE II

Effects of varying concentrations of representative monocarboxylic acids when mixed with tumor cells prior to inoculation of mice*

Tumor cell	Monocarboxylic acid	Concentration (mg per ml)	Average survival (days)		Survivors (90 days)
			Test	Control	
6C3HED	Caproic	0.4	12	11	0/10
		0.8	25†	11	6/10
		1.6	∞‡	14	10/10
Ehrlich	Caprylic	0.8	15	13	0/10
		1.6	42	13	8/10
		2.5	∞‡	13	10/10
TA_3	Capric (decanoic)	0.5	17	14	0/10
		1.0	28	14	7/10
		2.0	∞‡	15	10/10

*All experiments were carried out at pH 4.5. Each mouse received 5 to 8 million tumor cells alone (control) or mixed with monocarboxylic acid (test).
†Calculated from the death times of the mice which failed to survive for 90 days.
‡Represents survival beyond the 90-day test period.

TABLE III

Difference in susceptibility of ascites tumors to monocarboxylic acids*

Monocarboxylic acid	Minimal effective concentration (m*M*)		
	6C3HED	Ehrlich	TA_3
Propionic (C_3)	21.6	43.2	43.2†
Butyric (C_4)	18.1	36.3†	36.3†
Valeric (C_5)	31.3	31.3†	31.3†
Caproic (C_6)	13.7	43.1	43.1
Caprylic (C_8)	5.5	17.3	5.5
Capric (C_{10})	11.6	11.6	11.6
Lauric (C_{12})	15.0	10.0	5.0
Myristic (C_{14})	4.3	4.3	4.3
Palmitic (C_{16})	7.8†	3.9	7.8†
Stearic (C_{18})	7.0†	7.0	7.0†

*All experiments were carried out at pH 4.5. Each mouse received 5 to 8 million tumor cells.
†Represents partial protection.

fication proved difficult to achieve at higher levels, experiments above the concentration indicated were not carried out. In tests with propionic, butyric, and valeric acids another type of difficulty was encountered. These three compounds proved to be readily soluble at all levels studied, but the relatively high concentrations required for activity against the Ehrlich and TA_3 ascites cells made it impossible to maintain the pH of the reaction mixtures above 3.5 without major alterations in the buffer system employed. Since pH values below 3.5 have been shown to be deleterious to the tumor cells (3), further experiments with these three fatty acids were not carried out.

Effect of pH on Activity of Monocarboxylic Acids

As noted in previous communications (1, 2, 3), the in vitro antitumor activity of the fatty acids tested was limited to a narrow range of pH values:

only mixtures at pH 4.5 or below proved to be effective against the ascites tumor cells. Each of the present series of monocarboxylic acids was tested over a wide range of pH values with the three experimental tumors. The same general limitation was found: antitumor activity could be demonstrated only at pH values below 4.5. However, one important exception to this general limitation was observed. Capric (decanoic) acid, alone of the monocarboxylic acids studied, was effective in suppressing the development of all three ascites tumors over a wide range of pH values. The results obtained with this fatty acid are summarized in Table IV. It is evident that decanoic acid, at concentrations comparable to that required for antitumor activity at acidic pH (Table III), is capable of suppressing the development of all three types of ascites tumors at the physiological pH of 6.8.

TABLE IV

Effect of capric (decanoic) acid when mixed with ascites tumor cells at pH 6.8 prior to inoculation of mice*

Tumor cells	Concentration (mg per ml)	Average survival (days)		Survivors (90 days)
		Test	Control	
6C3HED	2.0	22†	13	3/10
	4.0	∞‡	18	10/10
Ehrlich	2.0	11	17	0/10
	4.0	∞‡	11	10/10
TA_3	1.0	14	14	0/10
	2.0	∞‡	15	10/10

*All determinations were carried out at pH 6.8. Each mouse received 5 to 8 million tumor cells either alone (control) or mixed with capric acid (test).
†Calculated from the death times of the mice which failed to survive for 90 days.
‡Represents survival beyond the 90-day test period.

Discussion

In previous investigations on the antitumor activity of fatty acids (1, 2, 3), two major drawbacks to therapeutic application were encountered. First, antitumor activity could only be demonstrated at pH values below 4.5 and, secondly, it was found necessary to mix the tumor cells with the fatty acids *before* inoculation into mice in order to achieve tumor suppression. The experiments reported in the present communication were devised to furnish additional data on the relationship between chemical structure and antitumor activity of fatty acids, and to continue the systematic search for fatty acids active at physiological pH. In the monocarboxylic acid series of chain length from C_3 to C_{18}, one such compound has been found, decanoic acid. This observation is deemed to be of considerable significance since the component of royal jelly exhibiting essentially all the antitumor activity of this material has been identified as 10-hydroxy-2-decenoic acid (1, 2).

In previous studies with the dicarboxylic acids (3), it was found that the antitumor activity increased progressively with increasing chain length of the fatty acids. In general, similar observations were recorded in the present study

with the saturated monocarboxylic acids. The greatest activity was found with compounds in the carbon chain length of C_8 to C_{14}, and tended to be less marked in compounds with carbon numbers above or below this range. Strict interpretation of this general observation is rendered difficult, however, by the marked variability of the three experimental tumors in their response to the individual fatty acids. The minimal effective concentrations of the monocarboxylic acids were found to be approximately equal to those of the dicarboxylic acids established previously (3).

The phenomenon of pH dependency in the antifungal activity of fatty acids is well known. Chattaway, Thompson, and Barlow (6) showed that the fungicidal activity of saturated fatty acids of chain length from C_2 to C_{14} increased with decreasing pH. Prince (7) concluded that the antifungal activity of undecylenic acid depended upon the undissociated molecule, since the fatty acid and its calcium salt showed equal activity between pH 4.5 and 5.0, but above this value the effective concentration was much higher in the case of the calcium salt. If the antitumor activity of fatty acids is at least partly dependent upon acidic pH, i.e., upon the undissociated molecule, then the members of the monocarboxylic acid series with higher chain lengths should show antitumor activity at higher pH values than those with less carbon atoms. Evidence to this effect was found previously among the dicarboxylic acids (3) and, in the present study, among the monocarboxylic acids. Thus, while propionic acid did not confer any protection above pH 3.9, lauric, myristic, and stearic acids gave positive results at pH 4.5. This finding is in agreement with the results of Hoffman, Schweitzer, and Dalby (8), who reported that while formic and acetic acids were fungistatic only at pH 2.0 to 3.0, fatty acids with three to six carbon atoms displayed activity at up to pH 5.0, and enanthic, caprylic, and pelargonic acids (C_7 to C_9) were fungistatic even at neutrality.

The present experiments do not explain the mechanism of the in vitro antitumor activity of fatty acids. It seems probable that the surface active properties of these compounds must play a major role in this activity. In addition, there is increasing evidence that activity is dependent, at least in part, upon the undissociated form of the molecule. Both these observations are of possible significance in view of the importance of dissociation properties and lipid solubility in the transport of drugs across cellular and intracellular barriers (9). Other factors which may have a bearing on the observed phenomena are the rapid oxidation of certain long-chain fatty acids by ascites cells (10, 11, 12) and the effect of fatty acids upon oxidative phosphorylation (11, 13).

The major finding of the present study has been the observation that capric (decanoic) acid, alone among the mono- and di-carboxylic acids tested, is effective in inhibiting tumor growth in vitro at the physiological pH value of 6.8. The minimal effective concentration of decanoic acid at pH 6.8 was found to be relatively low (2.0 mg per ml for TA_3 cells, and 4.0 mg per ml for 6C3HED and Ehrlich cells), and this would appear to indicate considerable tumor-suppressing ability. The discovery of a fatty acid with in vitro anti-

tumor activity demonstrable at physiological pH removes a serious drawback to possible therapeutic application of these studies.

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THE ACTIONS OF METABOLIC SUBSTRATES AND INHIBITORS ON THE RABBIT AURICLE*

BY

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Knowledge of cardiac muscle metabolism has advanced during the past few years to an appreciable extent, but the correlation of metabolic processes with the various functions of the heart has been very little investigated. It is possible that some of the enzyme systems that are highly active in cardiac extracts or homogenates, or even in heart slices, are not intimately involved in the beating of the heart. It is also possible that in the intact beating heart there are enzyme systems of great importance, which are either destroyed or altered by any damage to the myocardium. Such systems may be brought to light by a more detailed analysis of the actions of metabolic substrates and inhibitors on the normally contracting cardiac tissue. For the understanding of drug actions on the heart, it is frequently necessary to correlate the metabolism with the functional aspects of impulse discharge, conduction, and contraction. The isolated rabbit auricle was used in the present work because both the amplitude of contraction and the rate of impulse discharge could be more accurately measured with it than with the entire heart where there occur problems of conduction block and complex contractile movement. It is also difficult to maintain normal conditions in the perfused heart, and the tissue soon becomes damaged, whereas auricles will beat regularly for many hours in the proper medium. This work was done in connexion with the problem of the action of acetylcholine on the heart and of how this action is influenced by the metabolic state of the myocardium. The results obtained with acetylcholine will be reported in a subsequent paper.

METHODS

The effects of metabolically important substances on the rabbit auricle were determined by addition of the substances to a bath in which the auricles were beating. Hearts from young rabbits were used, inasmuch as it was found that such preparations beat more regularly and give more reproducible results than those from older rabbits. It is possible that in older animals the auricular walls are so thick that diffusion of oxygen into the tissue, or diffusion of metabolic products from the tissue, is not adequate. It also seemed that better results were obtained when the rabbits were allowed to remain quiet for several minutes before they were killed. Auricles obtained from animals that had been killed immediately after being caught and handled often gave poor results

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and would seldom achieve a regular beat. The rabbits were killed by a blow on the head and the hearts were rapidly removed. The hearts were allowed to beat for about half a minute in a dish of physiological medium in order to allow as much blood as possible to be pumped out of the tissues. The ventricles were often massaged in order to rinse the blood from the auricles, care being taken not to touch the auricles. The auricles were then cut from the heart and carefully trimmed so that no ventricular tissue remained. Caution was exercised in the trimming of the arteries and veins that no damage be done to the adjacent regions of the auricle, for it is in these regions that the pacemaker tissue usually lies. The auricles were suspended in 70 ml. of Tyrode solution (0.137 M-NaCl, 0.0027 M-KCl, 0.0018 M-$CaCl_2$, 0.00011 M-$MgCl_2$, 0.012 M-$NaHCO_3$, 0.00042 M-NaH_2PO_4, and 0.0055 M-glucose) which, after saturation with the gas mixture, had a *p*H of 8.1. Substances to be tested were added in 0.7 ml. volumes at a concentration 100 times that required in the final medium. Although this medium is a good buffer, all solutions of these substances were brought to a *p*H close to 8.1 before their addition to the bath. All measurements were made at 30° C. It was found that better results could be obtained at this temperature than at 38° C. At the higher temperature it is likely that the rapid rate often limits the recording of the full amplitude and this would introduce serious errors into the determinations of amplitude effects. The auricles also beat regularly over a much longer period of time at the lower temperature. The medium was continually saturated with a mixture of 5 per cent CO and 95 per cent O_2. The substances used were obtained in as pure a form as possible. The sodium fluoroacetate was kindly supplied by Dr. Saunders, of Cambridge University.

In order to achieve quantitative results the auricular tracings were carefully measured, the amplitude being recorded in millimetres and the rate of beating in contractions per ten seconds. It was necessary to use this small time unit for measuring the rate inasmuch as rapid rate changes could not have been accurately determined if longer intervals had been chosen. The effects produced by the addition of any substance were expressed in terms of the percentage change in the amplitude and rate. Such results might conceivably be expressed as absolute changes, percentage changes, or as final levels of activity to which the auricles were brought by the substances. For most purposes the percentage change is the most significant. In the first place, owing to the recording arrangement, the true amplitude of contraction is not measured; hence, if absolute changes were given, it would not be possible to compare one experiment with another. Furthermore, in considering the effects of substances on the auricular frequency we are usually interested in the changes in the rates of processes occurring within the pacemaker cells. It is likely that the rates of such processes are roughly proportional to the frequencies of beating. In metabolic studies the effect of an inhibitor on an enzymatic system is usually expressed in terms of percentage inhibition, and so here we might expect a more direct relationship to the underlying processes if we also express the frequency changes in similar terms. Justification for this method of expression, with regard to both rate and amplitude, was found in the quantitatively comparable results obtained from auricles beating at greatly different rates and amplitudes.

The auricles usually beat somewhat irregularly for 5–30 minutes after placing them in the bath, but eventually became quite constant with respect to both rate and amplitude. Experimental work was always initiated after the auricles had reached this steady state. Such auricles would beat for two to three days, the amplitude gradually decreasing during the second and third days. No auricles were considered to be normal after the first day and all results reported were obtained on fresh auricles. The recorded amplitude was usually adjusted to between 40–60 mm. Normal auricles beat spontaneously at an average rate of 102 contractions per minute, although there was quite a large variation between the auricles from different animals in this respect, the extremes recorded being 72 and 144. At 38° C. the frequency is approximately 80 per cent

gre[illegible]han at 30° C., and thus the rate at body temperature would have been approxi-ma[illegible]84 beats per minute, a figure not far distant from the normal *in situ* rate of the ra[illegible]art.

Results

The effects of metabolic substrates on normal auricles

[illegible] obvious that if a substance is to alter the beating of the auricles by acting thr[illegible] metabolic mechanisms, its quantitative effect will depend on the metabolic sta[illegible] the myocardial cells. If a particular substrate or intermediate, which is of [illegible]tance in the functional metabolism of the heart, is deficient, we might expe[illegible]ts addition to produce an effect. Whether this would be stimulation or depre[illegible]ion cannot be predicted until the correlation between the various metabolic reac[illegible]s and the functioning of the heart cells is better understood. On the other han[illegible]an enzyme system is saturated with respect to its substrate (that is, if the sub[illegible] is not limiting the rate of the reaction), there may be no response to the addi[illegible] of the substrate, or there may perhaps occur a retardation of the enzyme syst[illegible]hat operate before the particular step in which the substrate is involved. Fur[illegible]ore, the observed actions of substrates on the auricular tissue may be ind[illegible]nt of enzymic mechanisms, the primary effect being upon the cellular mem[illegible] potential or the contractile substance itself. Lastly, the possible actions of [illegible]abstances on the acetylcholine equilibrium in the cardiac tissue must be bor[illegible]mind. Since there is a slow formation of acetylcholine in the auricles feiti[illegible]thin the heart cells themselves or released from the post-ganglionic nerve endi[illegible] it is possible that a substance may stimulate or depress this formation, or af[illegible]he rate at which this endogenous acetylcholine is destroyed by modifying the a[illegible]y of the cholinesterase, or antagonize the action of the acetylcholine on the a[illegible]ular cells.

[illegible]esults obtained on the actions of substrates on normal auricles are summ[illegible]zed in Table I. Changes of less than 5 per cent are probably not significant unless derived from experiments upon several auricles. An inspection of this Table will show that stimulation of either rate or amplitude is very seldom seen with any of the substrates investigated. It is likely that the only really significant stimulation is that produced by citrate on the rate. These substances, in general, have no effect on the normal auricle until a certain concentration is reached and then they begin progressively to depress it. This is seen in the concentration-action curves in Fig. 1. On the whole, it is probable that the enzymatic systems involved in providing energy for the beating of the auricles are not limited by any of the substrates tested.

The amplitude is more readily depressed by these substances than the rate. On the average, for any specific concentration of substrate, the depression of the amplitude is two to three times as great as the depression of the rate. However, substrates vary in this respect and it will be seen that acetate at lower concentrations depresses the rate as strongly as it does the amplitude. The maximum effect, in most instances, is reached from one to two minutes after the addition of the substrate to the bath. There is then, with the majority of substrates used, a partial or complete spontaneous recovery from this depression. This was observed with pyruvate, lactate, fumarate, malate, acetate, oxalacetate, propionate, and butyrate; it was not seen with citrate or caprylate, the depression produced by these being

TABLE I

THE EFFECTS OF METABOLIC SUBSTRATES ON NORMAL AURICLES

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Succinate	0.0001	1	0	0	1	1
		5	0	+2	1	1
	0.001	1	0	0	1	1
		5	0	+3	1	1
	0.005	0.5	0	+7	1	1
		1	0	+9	2	5
		2	0	+12	2	4
	0.01	0.5	+18	22	3	3
		1	13	26	4	4
		2	10	27	3	3
Fumarate	0.001	0.5	6	4	1	1
		1	6	5	1	1
		2	3	0	1	1
	0.005	0.5	3	5	2	2
		1	+3	7	3	3
		2	0	+6	2	2
	0.01	0.5	+9	+8	2	3
		1	+5	+14	3	4
		2	+2	+10	3	3
Malate	0.001	0.5	+3	0	2	2
		1	3	0	2	2
		2	6	1	1	1
	0.005	0.5	+2	+10	3	6
		1	+4	+14		5
		2	0	15	3	3
	0.01	0.5	4	9	2	2
		1	+4	+18	2	3
		2	+4	+20	2	2
		5	0	+15	2	2
		10	0	8	2	2
		15	0	+5	1	1
	0.02	0.5	+7	+23	1	1
		1	+14	32	1	1
		2	+7	27	1	1
		5	0	+22	1	1
		8	0	12	1	1
		15	0	+8	1	1
Oxalacetate ..	0.002	0.5	0	2	1	1
		1	0	2	2	2
		2	0	0	1	1
	0.01	0.5	0	9	1	1
		1	0	+14	1	1
		2	0	13	1	1
		3	0	11	1	1

TABLE 1—*contd.*

Drug	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Pyruvate	0.0001	1	0	0	2	2
		2	0	−2	2	2
	0.001	0.5	0	−2	2	2
		1	0	−5	2	2
		2	0	−6	2	2
		5	0	0	2	2
		10	0	+3	2	2
	0.005	0.5	−1	−3	5	10
		1	−1	−6	7	13
		2	−1	−11	5	10
		3	−2	−9	3	5
		5	−2	−8	4	6
		10	−4	−5	3	3
		15	0	−3	2	2
	0.01	0.5	0	−9	2	2
		1	0	−12	4	4
		2	0	−16	5	5
		3	0	−7	2	2
		15	+5	−6	1	1
	0.02	0.5	0	−8	1	1
		1	−8	−3	1	1
		2	0	−23	1	1
Acetate	0.00002	1	0	0	1	1
		5	0	0	1	1
	0.0001	1	−6	−8	1	1
		2	0	4	1	1
	0.0005	0.5	9	4	2	3
		1	−22	−15	2	3
		2	−17	−10	2	3
	0.001	0.5	5	9	4	5
		1	19	21	3	4
		2	−11	−9	3	3
		5	−3	2	2	3
	0.01	0.5	−4	8	2	2
		1	−6	−23	3	4
		2	−4	−16	2	3
		5	0	−7	2	3
		10	+6	−3	2	2
		15	+6	−2	2	2
	0.03	1	6	27	2	2
		2	−4	22	2	2
		5	0	−18	1	1
		10	+6	11	1	1
		15	+6	15	1	1

TABLE I—*contd.*

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Propionate ..	0.0005	0.5	0	−1	2	2
		1	0	−4	2	2
		2	0	−3	2	2
		5	+3	−8	2	4
		10	+7	−3	2	3
	0.005	0.5	−7	−10	1	2
		1	−5	−7	1	2
		2	0	−3	1	2
		5	+5	−1	1	2
	0.01	0.5	−7	−4	1	1
		1	−7	−6	1	1
		3	0	−3	1	1
Butyrate	0.0005	1	0	−1	1	1
		5	0	0	1	1
	0.001	1	0	−10	1	2
		2	−3	−8	1	2
	0.005	1	0	−16	1	2
		2	−6	−11	1	2
		5	−3	−8	1	2
	0.01	0.5	0	−7	1	1
		2	0	−6	1	1
β-Hydroxy-butyrate	0.001	0.5	0	−4	1	[illegible]
		1	0	−6	1	[illegible]
		3	0	−10	1	[illegible]
	0.005	1	−7	−11	1	[illegible]
		3	0	−13	1	[illegible]
Caprylate	0.00002	0.5	0	0	1	[illegible]
		1	0	0	1	[illegible]
		3	0	−4	1	[illegible]
		5	0	−9	1	[illegible]
	0.0001	0.5	0	0	1	[illegible]
		1	0	−5	1	[illegible]
		3	−6	−16	1	[illegible]
		5	−6	−26	1	[illegible]
	0.0005	0.5	−6	0	1	[illegible]
		1	−17	−11	1	[illegible]
		5	−6	−24	1	[illegible]
	0.001	1	−7	−15	1	[illegible]
		2	−14	−25	1	[illegible]
	0.01	1	−22	−33	1	[illegible]
		2	−22	−42	1	[illegible]

TABLE I—*contd.*

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Glu[illegible]e ..	0.001	1	0	0	1	1
		2	0	0	1	1
	0.005	1	0	0	1	1
		2	0	0	1	1
	0.01	1	0	−3	2	3
		2	0	−1	2	3
	0.03	1	0	−7	1	1
		2	0	−5	1	1
Lactat[illegible]	0.001	1	0	−2	1	1
		2	0	−1	1	1
	0.005	1	0	−1	2	2
		2	0	−1	2	2
	0.01	1	0	−6	2	2
		2	0	−2	2	2
		5	0	0	2	2
	0.03	1	0	−9	1	1
		2	0	−3	1	1
		5	0	−2	1	1
Cit[illegible]	0.0001	1	0	0	1	1
		2	0	0	1	1
	0.001	0.5	0	−5	2	3
		1	−2	−11	2	3
		2	−2	−12	2	3
	0.005	0.5	0	−28	2	3
		1	+3	−47	2	3
		2	+6	−60	2	3
		4	+2	−62	2	3
	0.01	0.5	0	−35	2	2
		1	+16	−63	2	2
		2	+25	−74	2	2
		5	+28	−75	2	2
		10	+25	−74	2	2
		15	+20	−72	2	2

irreversible until the substance was washed from the bath. Typical curves illustrating this spontaneous recovery are given in Fig. 2. This recovery is not due to destruction of the substrate, for in most instances the total amount of substrate present in the bath is sufficient to prevent any appreciable decrease in the concentration resulting from amounts destroyed by the tissue in the short time intervals involved. This type of spontaneous recovery is seen in cardiac tissue under the influence of a variety of substances, and is probably to be interpreted in the light of the ability of the cardiac functional systems to adjust themselves to abnormal conditions.

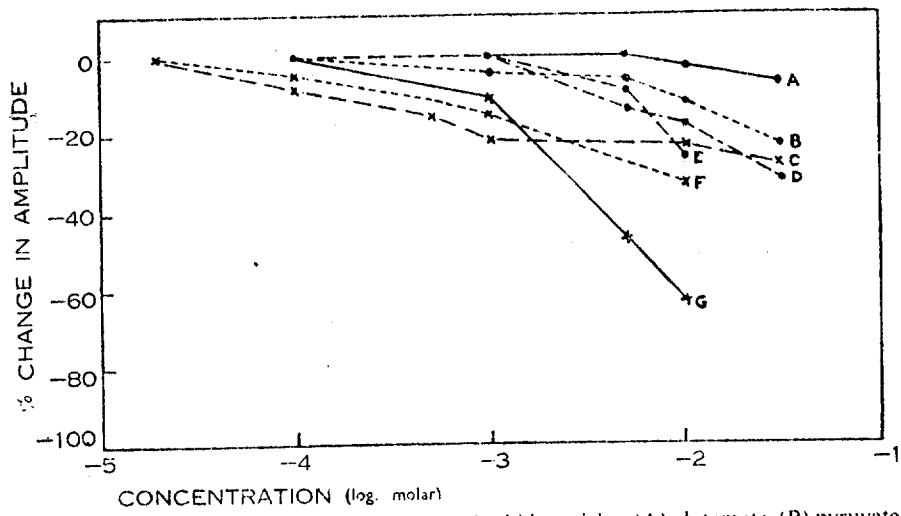


FIG. 1.—The action of substrates on the normal rabbit auricle. (A) glutamate, (B) pyruvate, (C) acetate, (D) malate, (E) succinate, (F) caprylate, (G) citrate. Time of action: 1 min.

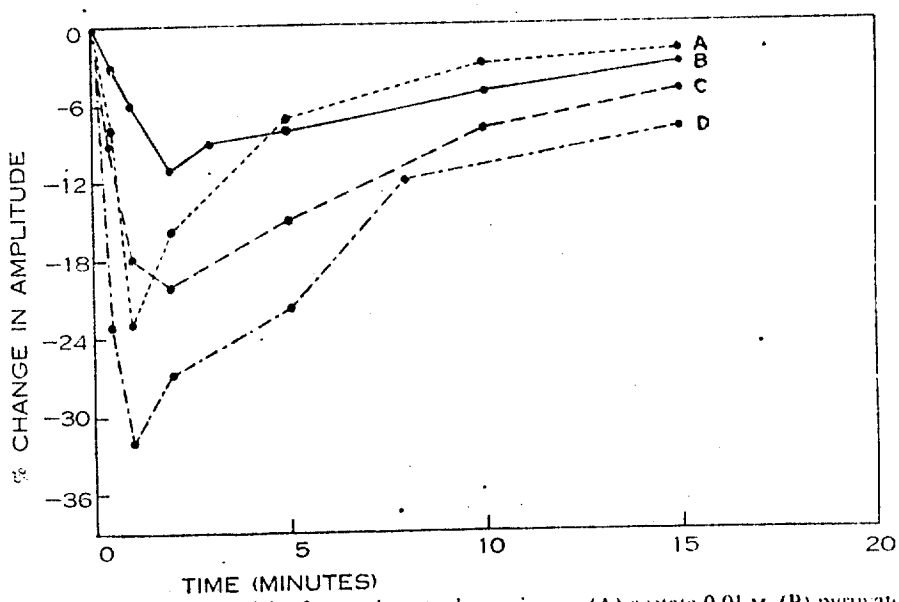


FIG. 2.—Recovery of auricles from substrate depression. (A) acetate 0.01 M, (B) pyruvate 0.005 M, (C) malate 0.01 M, (D) malate 0.02 M.

Consideration of the penetration of these substances into the cardiac cells is of the greatest importance, for, unless the heart cells, like some bacteria, possess enzymatic systems upon their surfaces, it is necessary for the substances to enter the cells in order to produce a direct effect upon the metabolism. Very little work has been done on the permeability of cell membranes to such substances. Further

mo[illegible] analogies between the penetration of these substances and other weak aci[illegible] be drawn with confidence, inasmuch as cell membranes seem frequently to [illegible]ess selective permeabilities for substances of functional importance. At the [illegible]tly alkaline *p*H of this medium, these substrates exist in solution preponderant[illegible] anions; this may interfere with rapid penetration, especially when the sub[illegible]es possess two or three such ionized groups. However, it must be borne in m[illegible]d that the permeability to charged molecules may markedly increase in the hea[illegible]ells during the passage of an impulse over the tissue. There is some evidence for the penetration of such substrates. It will be shown later that in substrate-depl[illegible]d auricles there is an almost immediate effect of added pyruvate. Furthermore the actions of pyruvate, lactate, β-hydroxybutyrate and other substrates are quite rapid on exhausted perfused hearts. It will also be shown that iodoacetate and [illegible]oroacetate penetrate the auricular cells quite readily. With regard to dicarboxy[illegible] and tricarboxylic acids, I know of no evidence that is applicable to the pres[illegible]situation, although it seems certain that succinate and malonate are able to pen[illegible]e into heart slices. Fig. 1 shows no correlation between effect and the num[illegible] of carboxyl groups. I believe that the evidence available points to a de[illegible] penetration by these substances. However, equilibrium may not be reached for [illegible] to three minutes, the times of maximum substrate action thus being expressiv[illegible] the relatively slow rate of penetration compared to uncharged molecules.

[illegible] possible that the effects observed with the substrates may have been partly or [illegible]lly non-metabolic effects. The relationship between cellular membrane pote[illegible]ls and the rate of discharge of the pacemaker cells or the degree of contraction [illegible] not understood, but it is conceivable that alterations brought about in the cell [illegible]embrane by these substrates might influence the auricular functioning. Such organ[illegible] acids are known to be capable of producing temporary electrical potential changes in nerve, muscle and plant cells. If such a mechanism of action is involved, it is likely that we must interpret the spontaneous recoveries from substrate depression as originating in the gradual restoration of the normal potential by the establishing of a concentration equilibrium between the medium and the interior of the cells. That these substrates depress the auricles by increasing in some manner the effective acetylcholine concentration in the tissue is unlikely, since it was clearly shown that atropine, at a concentration capable of blocking all acetylcholine action (1×10^{-8}), failed to alter the actions of pyruvate, citrate, acetate, malate, and fumarate. It is improbable that the depressant action originates in a simple increase in osmotic pressure of the medium, for glucose may be added up to a concentration of 0.03 M without alteration of the beat. Furthermore, if an osmotic effect were involved, we should expect the various substrates to produce similar degrees of depression at equivalent concentrations; this, however, is not the case. Since the concentration-action curves for the various substrates, as shown in Fig. 1, are not only at different positions along the concentration axis, but also possess markedly different curvatures, it would seem unlikely that the depressant effects were due to any general, non-specific type of action.

Some of the substrates, particularly acetate, malate, and propionate, showed a tendency to induce arrhythmias in the auricles. These arrhythmias were occasionally completely irregular, but usually they were of the "pulsus alternans" type, the amplitude of contraction regularly alternating each beat between two different

values. Sometimes such a rhythm was caused by the regular beating of one auricle of a pair while the other auricle beat only every other contraction, but more frequently this was not so. Rapid recoveries from such arrhythmias were observed when the substrate was removed from the bath.

The action of succinate was only depressant in these experiments. Depression of the amplitude was observed by Salant, Livingston, and Connet (1917) on the perfused frog heart (succinate 0.01–0.02 M) and by Forssmann and Lindstén (1946) on the isolated and perfused rabbit heart (succinate 0.01–0.04 M). They did not find in these preparations such marked depression of the rate as was here observed on the rabbit auricles. Forssmann and Lindstén (1946) found a mild stimulation (+ 15 per cent) of the amplitude by succinate in lower concentrations (0.0005–0.005 M). In the auricles no such stimulation was observed within a similar concentration range; this possibly indicates a difference in behaviour between the auricles and ventricles of the rabbit, inasmuch as they measured the contractions of the left ventricle. No stimulation was found in the isolated and perfused rat heart (Nakamura *et al.*, 1949). Despite the rapid oxidation of succinate by cardiac tissue, it would appear that the energy derived from this is, at least in the normal auricle, of no consequence in the functioning of the auricles. It will be shown later that this energy cannot be utilized to revive substrate-depleted auricles.

The relatively marked depression produced by malate on the auricles is in accord with the results of Salant, Livingston, and Connet (1917) on the frog heart; the frog heart would seem to be more sensitive to the action of this substrate than the rabbit auricle. A definite difference in the behaviour of the two tissues is seen however, in the recovery of the rabbit auricles from the malate depression, whereas the frog heart was reported to remain in a constant state of depression as long as the malate was present (in one case for 62 minutes).

The action of citrate on the rabbit auricle is very interesting, there being a marked stimulation of the rate while the amplitude is simultaneously reduced. This phenomenon occurs only at higher concentrations of citrate (above 0.005 M); below this there is little effect on the rate, although the amplitude is depressed. The acceleration of the rate occurs quite suddenly. For example, in one experiment using 0.01 M-citrate, the rate was unchanged 30 seconds after the addition of the citrate, but it suddenly increased by 22 per cent at 40 seconds, and was up by 39 per cent at 60 seconds, after which it remained relatively constant. The depression of the amplitude observed here is very similar to that reported by Forssmann and Lindstén (1946) on the entire heart, but, unfortunately, they did not include their results on the rate changes. How much of the action of citrate was due to a decrease in the concentration of ionized calcium in solution is difficult to say. Added calcium will counteract the depression produced by citrate, but this does not prove that citrate acts by calcium depletion. When the concentration of citrate is 0.001 M or above, there must be a decrease in the ionized calcium, the original concentration of the calcium ion in the medium being 0.0018 M. If one considers the effect on the rabbit auricle of decreasing the calcium concentration (Webb, 1950), and compares this with the concentration-action curve for citrate depression of the amplitude one must admit that the results coincide with what one might expect if citrate were acting by calcium depletion. On the other hand, the stimulation of the rate cannot be due to a decrease of ionized calcium in the medium, for low calcium concentra-

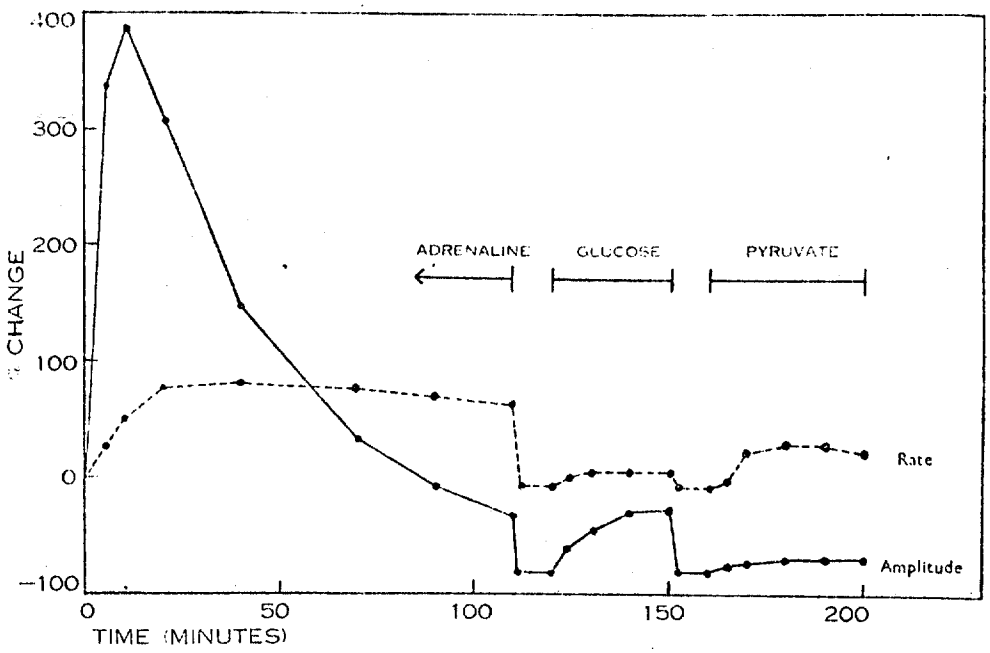
[illegible] ive very little, if any, effect on the rate of the rabbit auricle. There still [illegible] the possibility that the citrate penetrated the pacemaker cells and within [illegible] oduced a disturbance in the calcium equilibrium, thereby producing changes [illegible] arge rate that would not have occurred as the result of a mere decrease in [illegible] calcium.

The effect of glucose deficiency on rabbit auricles

[illegible] abbit auricles were left for 24–48 hours at 30° C. in a glucose-containing [illegible] a marked reduction in the amplitude was usually observed, especially [illegible] medium had been continually replaced, although the rate often remained [illegible] as it was originally. Under such circumstances the auricles stop in [illegible] xed condition of diastole. These auricles could usually be restarted with [illegible] holine or adrenaline (Vane, 1948; Burn and Vane, 1949) but not by any [illegible] substrates that were tried (pyruvate, acetate, succinate, oxalacetate, and [illegible]). This type of exhaustion occurs in the presence of glucose and is probably [illegible] ed in any way to depletion of substrate. It is likely that some alterations [illegible] cellular enzyme systems have taken place, such as the depression of the [illegible] n of acetylcholine (Bülbring and Burn, 1949) or a dissociation of the [illegible] of oxidation and phosphorylation.

[illegible] ucose was removed from the medium in which the auricles were beating, [illegible] s no immediate effect. It is to be expected that the auricles would have [illegible] endogenous substrate—glycogen and fat—on which to operate for a period [illegible] The amount of work performed by beating auricles under these conditions [illegible] great, for they were not working against the usual internal pressure of the [illegible] Seven experiments were performed in which the behaviour of the auricles [illegible] absence of glucose was observed for five to seven hours. In three of these [illegible] ents there was no change of rate, and in four there was some decrease [illegible] 10 to –30 per cent). In three of these experiments there was no change in amplitude, and in four there was some decrease (–30 to –50 per cent). The auricles thus show variation in their behaviour in the absence of glucose, some apparently having a greater supply of endogenous substrate than others. Since there was essentially no change in rate or amplitude over a period of five to seven hours in the presence of glucose, these decreases in rate and amplitude, when they occurred, must be attributed to the lack of glucose. In longer experiments, when the auricles were left without glucose overnight, the exhaustion produced by substrate depletion is complicated by the simultaneous development of the type of exhaustion seen when glucose is present. Furthermore, it seems evident that lack of glucose in the medium produced some irreversible change or damage in the cardiac cells, for although stimulation was observed when glucose was added to such auricles, there was never anything like complete recovery. This type of damage may be related to that occurring when cardiac tissue is subjected to oxygen deficiency (Webb, Saunders, and Thienes, 1949a).

In order to produce rapid substrate depletion, with as little complication from other changes as possible, the auricles were driven with adrenaline (1×10^{-6}) in several experiments for varying periods of time in the absence of glucose. Such a procedure exhausted the auricles to a low level of activity in 15 to 60 minutes. If adrenaline were allowed to act for one to three hours, the auricles, after the



IG. 3.—Substrate depletion of the auricles and the actions of glucose and pyruvate. Average of four experiments. Adrenaline, 10^{-6}; glucose, 0.0055 M; pyruvate, 0.005 M.

adrenaline had been washed out, beat with a very small amplitude, although the rate was usually relatively unaffected. The averaged results of four experiments are shown in Fig. 3. The rate in the presence of adrenaline remained at a high level, while the amplitude rapidly decreased to below the normal level. After the adrenaline had been removed, the rate returned to nearly its original value and the amplitude was very depressed. Exhaustion with adrenaline in the absence of glucose seems to affect the amplitude much more than the rate, the latter being quite stable. In spontaneous exhaustion in the absence of glucose the amplitude was also more easily depressed. The question whether adrenaline produces cardiac failure by depletion of substrate under such circumstances has never been settled. Some investigators have found that adrenaline-driven hearts lose their glycogen and attribute the failure to this, while others have shown that the heart may fail when there is sufficient glycogen present (Chang, 1936; Fieschi, 1933; Bogue, Evans, and Gregory, 1937; and others). This problem might be solved by determining the extent to which the cardiac tissue will recover when glucose is given. If there is complete, or nearly complete, recovery the failure may be attributed to substrate depletion, while if no recovery is seen it is likely that damage and disorganization have occurred to the functional systems. Any intermediate situation would indicate the extent of substrate depletion or cardiac damage. In the present investigation we shall see that the situation is intermediate and we must conclude that changes have taken place in the auricular tissue in addition to substrate depletion. However, for convenience we shall speak of such auricles as depleted.

en the auricles were driven with adrenaline there was invariably a gradual de ment of contracture as the amplitude decreased. This may also be ex d as an increase in tone or a decreased ability of the auricular muscle to x. Auricles brought to almost complete exhaustion with adrenaline in the ence of glucose usually beat at a normal rate with a greatly diminished am de and were in a state of contracture. This inability to relax was also see the longer experiments when glucose was absent and no adrenaline was use ut it was never observed in exhaustion occurring when glucose was present. The problem of relaxation will be discussed after the results obtained with various met lic inhibitors have been presented.

Effects of metabolic substrates on substrate depleted auricles

Auricles that had been spontaneously depleted or driven with adrenaline in the sence of glucose were invariably stimulated by the addition of 0.0055 M glu , the concentration normally present in Tyrode solution. Furthermore, the de of contracture of the auricles was lessened; that is, the auricles showed an ased ability to relax. Sometimes the degree of relaxation could be alternate ncreased and decreased by additions and removals of glucose. Complete re to the initial amplitude was never seen, but the rate, if it was decreased, w quently restored to its normal value. The rate, however, in the depleted au was never far below its original value, while the amplitude was markedly dep d to only 10–20 per cent of the initial height. Usually there was a fairly rap e in amplitude over the first two minutes after glucose addition, and this was llowed by a more gradual increase to reach a maximum value after 10–40 min . In terms of percentage, glucose increased the amplitude by 100–300 per cent ver the depressed value. This is illustrated in Fig. 3, where the action of glucose may be compared with that of pyruvate.

Pyruvate likewise stimulated the substrate depleted auricles but not so well as glucose. However, the effect upon the rate was more marked with pyruvate, the rate being occasionally increased over the normal original value, while a good effect was sometimes obtained on the rate with little or no stimulation of the amplitude. The stimulation of the amplitude by pyruvate was usually between 10–60 per cent, and when compared with glucose on the same auricles this amounted to about one-fifth of the glucose effect. Pyruvate also induced relaxation of the auricles but was not so effective as glucose in this respect. The optimal concentration of pyruvate for such stimulation was found to lie between 0.001–0.005 M.

Only acetate and β-hydroxybutyrate of all other substrates tested were capable of producing any recovery in the substrate depleted auricles, and these effects were small and variable. Neither of these substances was found to induce relaxation. With acetate, the concentration had to be low (0.001 M) for any stimulation to be observed, for if higher concentrations were used only further depression resulted. In six experiments with 0.001 M-acetate, the average increase in amplitude was 18 per cent whereas the rate was not stimulated. Substrates with no ability to revive depleted auricles were fumarate, succinate, citrate, malate, oxalacetate, propionate, butyrate, and glutamate.

It is interesting to note that glucose and pyruvate would occasionally facilitate relaxation of the auricular muscle although the auricles were not beating. Here we may confidently say that depletion of substrate increases the tone of the cardiac muscle and that the presence of glucose or pyruvate decreases the tone. When the auricles were beating, the amplitude of contraction usually decreased while the tone was increasing, and vice versa, but this was not always true, and we must conclude that there is no necessary relationship between the degree of contraction, and the ability of the muscle to relax. Failure of the muscle to relax sufficiently naturally inhibited the development of a full contraction, but it was observed occasionally, when glucose or pyruvate were added while the contracture was proceeding, that the amplitude may increase without simultaneous relaxation.

The results presented here on the rabbit auricle agree well with those obtained in the heart-lung preparation and in the isolated and perfused mammalian heart, not only with respect to the substrates that are utilizable by the cardiac tissue, but also with the comparative abilities of these substrates to revive the depleted heart muscle. However, on no other tissue are the differential actions of glucose and pyruvate on the rate and amplitude so easily observed. On may conclude that auricular and ventricular functional metabolism is qualitatively similar. It is likely, however, that somewhat different systems are involved in the discharge of impulses by the pacemaker cells and in the processes of contraction.

Effects of metabolic inhibitors on normal auricles

Curves showing the relationship between the concentration of inhibitor and the action produced are presented in Figs. 4 and 5. The time course of inhibit

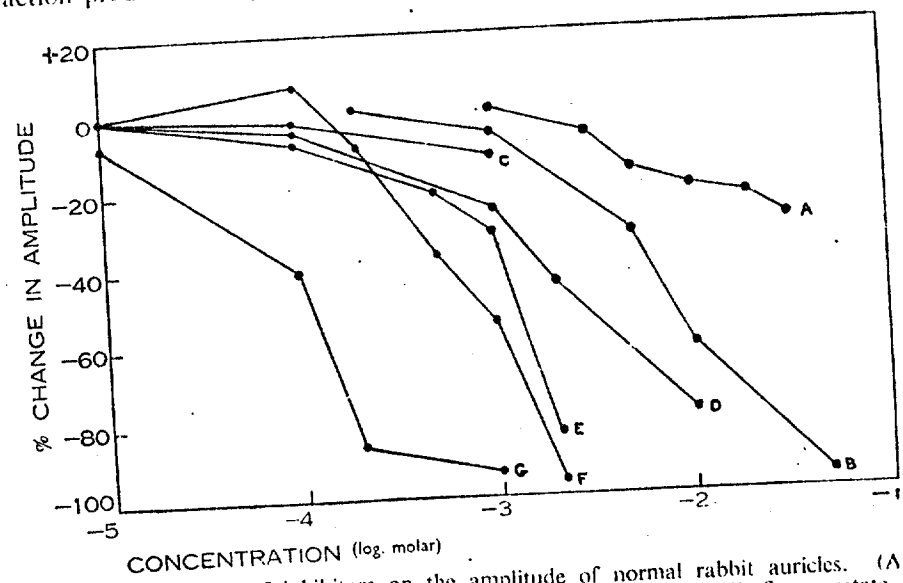


FIG. 4.—The action of inhibitors on the amplitude of normal rabbit auricles. (A) malonate—2 min., (B) fluoride—4 min., (C) phlorizin—5 min., (D) fluoroacetate—25 min., (E) azide—4 min., (F) cyanide—2 min., (G) iodoacetate—25 min. The times refer to the duration of inhibitor action.

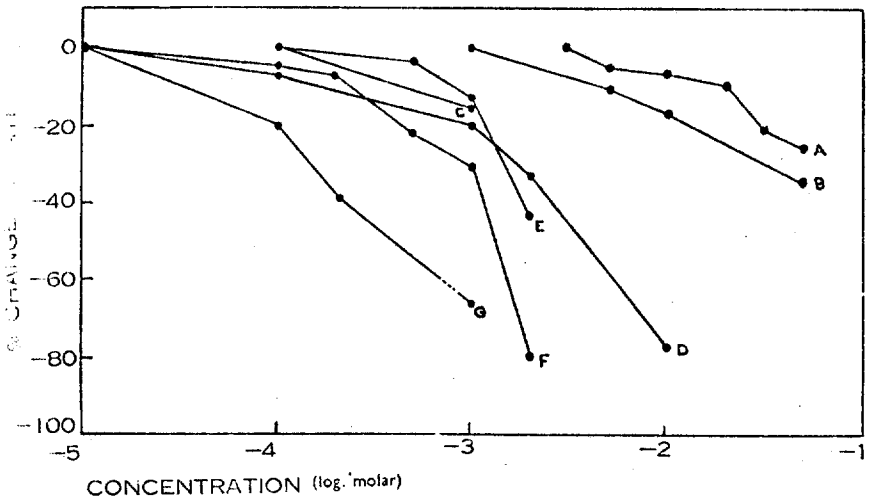


Fig. 5.—The action of inhibitors on the rate of normal rabbit auricles. Same designations as in Fig. 4.

[illegible] is illustrated in Figs. 6 to 12, where the actions upon rate and amplitude [illegible] be compared. These are all average curves obtained from three to six [illegible]ments.

C[illegible]de

[illegible]anide invariably produced a temporary stimulation before it began to depress th[illegible]uricles, this stimulation being more marked in the amplitude than in the rate. Th[illegible] degree and duration of stimulation varied with the concentration of cyanide: at low concentrations (0.00001–0.0001 M) the stimulation was small but persisted, often not being followed by a depression, but as the concentration was increased the period of stimulation became shorter and shorter, so that at concentrations around 0.001 M the maximum stimulation was reached 20–40 seconds after addition and was followed immediately by a rapidly developing depression. Such a temporary stimulation was also seen when the auricles were made anoxic.

The rapid development of the depression would indicate that the available supply of high-energy phosphate bonds in the auricle, at least with respect to some process, is not great, unless cyanide, through some unrecognized action, prevented the direct utilization of this energy. A concentration of cyanide of 0.001 M produces a final depression of about 90 per cent in the amplitude and 50 per cent in the rate; this concentration was reported to inhibit the respiration of minced rabbit heart 45 per cent (Banga, Schneider, and Szent-Györgyi, 1931) and the respiration of rat heart slices 74 per cent (Webb, Saunders, and Thienes, 1949b). The rabbit auricle will usually stop in a concentration of 0.002 M-cyanide. It is probable that, when the auricles stop, there is still a definite fraction of the respiration proceeding; the level of activity to which the auricles are brought by any concentration of cyanide is not a quantitative measure of the cyanide-resistant respiration. Impulse discharge from the pacemaker cells was not inhibited so readily as the amplitude of contraction; this has been observed with other cardiac

preparations (Loewi, 1897; Beresin, 1913; and others). Most workers have reported that hearts poisoned by cyanide stop in diastole (Loewi, 1897; and others) but some have observed systolic standstill (Fujimaki, 1924). In the rabbit auricle both may be observed according to the conditions of the inhibition. When the auricles were stopped rapidly by higher concentrations of cyanide, they stopped in the relaxed condition of diastole; however, if the action of cyanide at concentrations just below those required to stop the auricles was prolonged, the auricles began to go into contracture and eventually stopped in systole. Occasionally both phenomena were observed in the same experiment. In one instance, the auricles were stopped in 80 seconds by 0.002 M-cyanide, but they slowly and partially recovered in the presence of cyanide, so that at 30 minutes the rate was depressed to only 40 per cent of its original value and the amplitude 92 per cent of its original value. At this stage the auricles were in a state of partial contracture, an inability to relax having been developed during this time. Eventually they stopped in systole. However, contracture is less commonly observed with cyanide than with inhibitors like iodoacetate, fluoroacetate, and fluoride.

Spontaneous recovery from depression in the presence of cyanide was very seldom observed, the example cited above being the only definite instance. Normally the auricles would reach a certain level of depression and remain there, or, if the depression were sufficient, they would slowly pass into a state of contracture. The auricular tissue seems to have very little ability to adjust itself to conditions in which the oxygen utilization is impaired; no ability to adjust to anoxic conditions was observed. During very slight cyanide depressions, there was occasionally some recovery, but this may be explained by loss of cyanide from the medium, some being carried off by the vigorous gassing. When the cyanide was removed from the medium, the auricles recovered completely, the rapidity of recovery depending on the concentration of cyanide and the duration of exposure. After marked depression following exposures of three to six minutes to 0.001 M-cyanide, the auricles would recover completely within ten minutes but if the exposure time was only one minute the recovery took place within three to five minutes. No irreversible damage to the cells was observed at these concentrations, unless full contracture was allowed to occur. After the cyanide had been washed out a stimulation of the rate during the recovery phase was frequently observed, amounting to a 20–30 per cent increase over the normal rate before the cyanide had been added.. Various arrhythmias commonly occurred during this recovery phase. It is possible that during the depression some energy containing or energy-supplying substance had accumulated in the cells and that when oxygen was again available this resulted in a temporary stimulation of activity; this stimulation, however, was observed only with regard to the rate.

When excess calcium was added to the medium there was a marked stimulation of the amplitude, the rate remaining unchanged (Webb, 1950). Cyanide (0.001 M) antagonized this action, so that lower concentrations of calcium (0.0001–0.0005 M) were almost without effect, while the action of higher concentrations was markedly diminished. Cyanide also diminished the stimulating action of adrenaline on the auricles. In one experiment, adrenaline (10^{-6}) normally increased the rate by 69 per cent and the amplitude by 390 per cent, but in the presence of cyanide (0.001 M) the observed effects of adrenaline were zero and 105 per cent respectively

A:

is interesting to compare the actions of cyanide and azide because of the usu accepted similarity of their enzymatic inhibitions. Azide was not as potent an ibitor of the auricles as cyanide. In equal concentrations, azide acted more slo than cyanide and at any particular time produced only 1/2 to 1/3 the dep sion that would have been produced by cyanide. In Figs. 6 and 7 comparison may made of their actions at 0.0005 M. Azide did not produce the temporary stim lation observed with cyanide, nor did low concentrations ever stimulate. As with cyanide, azide depressed the amplitude more than the rate. This was also observed on the isolated hearts of cats and rabbits by Graham (1949).

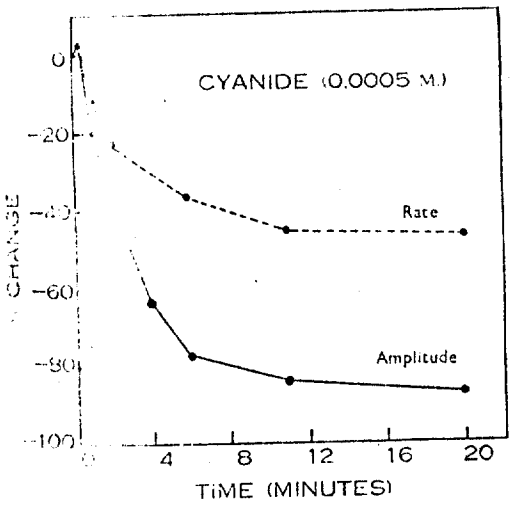


FIG. 6. The action of cyanide on the rabbit auricle.

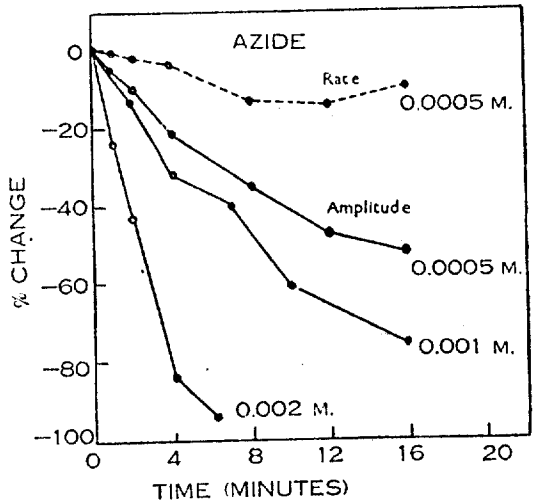


FIG. 7.—The action of azide on the rabbit auricle.

Recovery of the auricles after the azide had been removed was slower than with cyanide; 10 minutes were required after an exposure of 12 minutes to a concentration of 0.0005 M, and 20 minutes after a four-minute exposure to a concentration of 0.002 M, indicating that the azide is more tightly bound than cyanide.

Armstrong and Fisher (1940) concluded that cyanide and azide acted differently on fish hearts because the cyanide-stable respiration was different from the azide-stable respiration. However, this may be merely an expression of the wider range of action of cyanide at higher concentrations, for it is known that cyanide above a concentration of 0.001 M begins to inhibit dehydrogenases in addition to its action on metal-containing enzymes. However, it is clear that even in lower concentrations there was some difference in behaviour between the two inhibitors in their actions on the rabbit auricle.

Fluoride

Fluoride at concentrations between 0.005 0.01 M first depressed both the rate and amplitude of the auricles, but after approximately five minutes the amplitude began to increase and eventually reached values above the normal original level

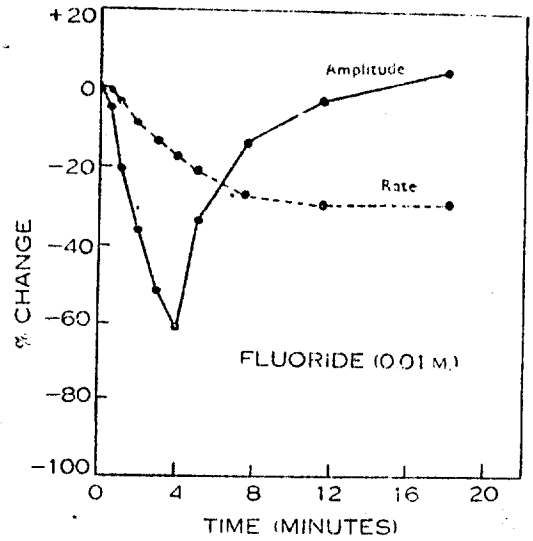


FIG. 8.—The action of fluoride on the rabbit auricle.

(see Fig. 8). Meanwhile, the rate remained at its depressed level or continued to decrease slightly. After 20–40 minutes the auricles were beating slowly but with a large amplitude. However, occasionally contracture began and the amplitude again decreased owing to the increasing inability of the auricles to relax. Previous reports on the action of fluoride on cardiac tissue have been equivocal. Several investigators have found only depression: Vernon (1910a, b) with 0.24–1.2 M-fluoride on the perfused tortoise heart, De Nitto (1928) with 0.00012–0.0012 M-fluoride on isolated mammalian hearts, and Chang (1938) with 0.0078 M-fluoride on rabbit auricles. Gottdenker and Rothberger (1935a, b) found depression of the perfused frog heart and the dog heart-lung by fluoride, but on guinea-pig and rabbit auricular strips they usually observed an increase in the amplitude when the fluoride concentration was 0.003 M. Ferrannini (1936) also found fluoride (0.0024–0.0072 M) to increase the force of contraction of rabbit auricular strips. In most work it has been observed that cardiac tissue is able to function surprisingly well in the presence of fluoride at concentrations which should inhibit glycolysis completely. Chang (1938) stated that fluoride, except for a slight depression, had little effect on the mechanic ability of rabbit auricles over a period of two to three hours as long as oxygen was present. Furthermore, he apparently observed no increases in amplitude. In the present work with the same tissue such a concentration of fluoride produced a marked effect. No explanation can be offered for these discrepancies.

With regard to the mechanism of fluoride action, most workers have concluded that depletion of calcium from the medium is not important. However, at least in the present experiments, it is difficult to see how it could fail to be of some importance. The calcium concentration in Tyrode solution is 0.0018 M; if sodium fluoride is added to the medium in a concentration of 0.005 M, there are approximately equivalent amounts of calcium and fluoride to form CaF_2. Since CaF_2 is soluble to the extent of 0.00022 M and since it is very little ionized (solubility product is 3.4×10^{-11}), it would seem likely that a definite fraction of the calcium would be withdrawn from the medium. When more fluoride is added the calcium should be reduced even more. No visible precipitation of CaF_2 was observed but the walls of the tissue bath slowly became covered with a white layer. Reduction of calcium in the medium leads to a marked depression of the auricle with respect to amplitude, but has no effect on the rate (Webb, 1950). Thus it would seem that the observed depression of rate and the subsequent increase

amplitude, in the presence of fluoride, must have been due to an action of fluoride other than that of altering the calcium concentration in the medium. As to how much the initial depression of amplitude is due to decreased calcium it is impossible to say.

Since fluoride inhibits cholinesterase, the possibility exists that at least part of the depressant action was due to prevention of destruction of endogenous acetylcholine. However, fluoride was found to give a typical depression of the auricles in the presence of physostigmine (10^{-6}), when the cholinesterase was already inhibited, and also in the presence of atropine (10^{-6}), when the action of acetylcholine was completely prevented. It is thus unlikely that the action of fluoride is related to the acetylcholine equilibrium in the auricles.

Such concentrations of fluoride as were found to have definite actions on the auricles (0.005–0.05 M) should inhibit glycolysis completely. Muscle enolase is usually inhibited 50 per cent by 0.001 M-fluoride and completely by 0.005 M-fluoride. A certain decrease in fluoride concentration would result from combination with calcium, but in the higher concentrations this could not have been very great. We must, therefore, conclude that the auricles can function quite well in the absence of the formation of phosphopyruvate from 2-phosphoglycerate. One possibility is that the auricles used fat for the necessary energy. Another possibility is that the 2-phosphoglycerate was utilized through some other system without the formation of pyruvate. It is interesting to speculate that the secondary increase in amplitude is due to this new source of energy, brought into play because of the enzyme block. In rat heart slices the respiration was never depressed more than [illegible] per cent by fluoride at concentrations as high as 0.1 M (Webb, Saunders, and [illegible]es, 1949b), so it is evident that in cardiac tissue there is an appreciable fraction of the metabolism insensitive to fluoride.

The fluoride inhibition is reversible, and after the fluoride had been washed out recovery was usually complete within 5–10 minutes. After long exposures there was occasionally some stimulation of the rate above the normal figure during the post-wash recovery stage. It was also noticed that the auricles during this period frequently had difficulty in relaxing and the tone varied correspondingly. In passing, it may be remarked that fluoride (0.03 M) quite effectively diminished the action of adrenaline (10^{-7}–10^{-6}), the stimulation of amplitude being almost abolished while the rate effect remained but was made weaker.

Iodoacetate

Iodoacetate in concentrations between 0.0001–0.001 M is a slowly acting depressant of the rabbit auricle, requiring from three to five minutes for appreciable reduction of the amplitude and from 10 to 20 minutes for a slowing of the rate. At certain stages of the depression, the amplitude was markedly reduced while the rate remained unaffected, but eventually both were depressed almost equally before the auricles stopped. During the later stages of this depression the auricles began to go into contracture and they stopped in systole, a phenomenon that has been invariably observed in work with iodoacetate (Weicker, 1934; Chang, 1938; and others). Although slight depressions produced by low concentrations of iodoacetate acting for short periods of time could be partially reversed by removing the iodoacetate, the usual result was that after the iodoacetate had been washed out the inhibition

proceeded, exactly as if the inhibitor were still present in the medium. This is, of course, what one would expect from considerations of the irreversible cellular reactions of iodoacetate, and it confirms previous results on other tissues and preparations.

Iodoacetate is not a specific inhibitor of glycolysis in mammalian cardiac tissue (Webb, Saunders, and Thienes, 1949b), and even in low concentrations it is likely that other enzyme systems are involved in its action. Not only is the synthesis of ATP diminished, by inhibition of the utilization of pyruvate and fatty acid breakdown products as well as by prevention of glycolysis, but it is likely that iodoacetate interferes with the utilization of ATP, for Buchtal, Deutsch, and Knappeis (1944) have reported that iodoacetate blocked the stimulating effect of ATP on skeletal muscle. It would not be surprising if iodoacetate reacted directly with myosin, since this protein complex possesses a number of sulphhydryl groups important to its functioning. This may explain the observation (Chang, 1938) that there is some ATP present in rabbit hearts that have been stopped by iodoacetate.

Adrenaline produced very little stimulation of the iodoacetate depressed auricle, and occasionally a reversal of the rate effect was observed when the depression was well advanced. In one typical experiment the concentration of iodoacetate was 0.001 M, the exposure time was 35 minutes, and the auricles were markedly depressed (rate −42 per cent, amplitude −96 per cent); adrenaline (1[illegible]) stimulated the amplitude slightly (28 per cent), but the rate was decreased to 75 per cent of the original value. It is not surprising that such auricles would fail to be stimulated by adrenaline, but the reversal of the rate effect was not expected. It is possible that iodoacetate blocks some step in the series of reactions through which adrenaline normally produces a stimulation of the rate, leaving the systems by which it may depress the rate at higher concentrations relatively unaffected.

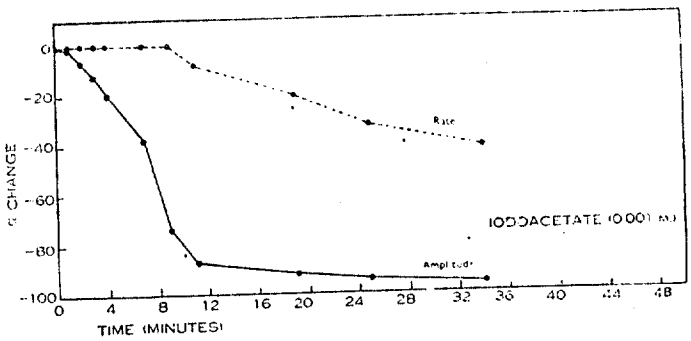


FIG. 9.—The action of iodoacetate on the rabbit auricle.

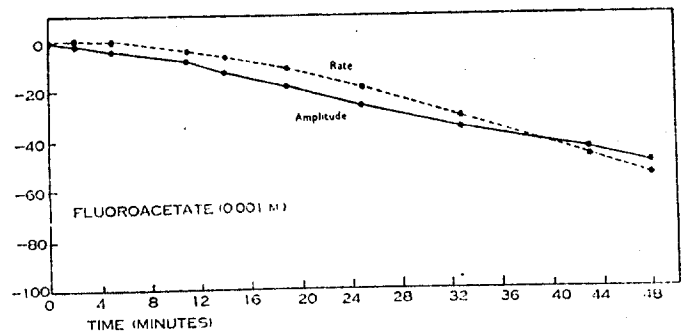


FIG. 10.—The action of fluoroacetate on the rabbit auricle.

Fluoroacetate

Fluoroacetate is the most slowly acting inhibitor of those investigated, depressing the auricles gradually and steadily for a period of two to three hours at a concentration of 0.001 M. It characteristically depressed both rate and amplitude equally, in this respect differing from any other inhibitor used in this study. The metabolic systems involved in impulse discharge and contraction may at some point possess a common path which is attacked by fluoroacetate. At the present time the exact mechanism of action of fluoroacetate is unknown, but it is likely that it inhibits some reaction, or reactions, in the tricarboxylic acid cycle. Accumulation of citrate and acetate during fluoroacetate action has been reported, but the depression of the auricles was not due to these intermediates, as may be seen by comparing the action of fluoroacetate with the actions of these substances. Furthermore, if such were the explanation, we should expect some degree of recovery when fluoroacetate was removed from the bath, but this did not occur. The action of fluoroacetate was quite irreversible. Occasionally the inhibition could be arrested by washing out the inhibitor, but the auricles never showed any tendency to return to their normal rate and amplitude.

Phlorizin

A nearly saturated solution of phlorizin in the medium (0.001 M) produced a slight depression of the auricles, the effect on the rate being somewhat greater than on the amplitude, the latter showing some ability to recover spontaneously in the presence of the inhibitor. The mechanism of action of phlorizin at these higher concentrations is not well understood, and therefore it is difficult at the present time to interpret these results or correlate the actions with any particular inhibition of enzymatic systems.

Pyrophosphate

Pyrophosphate at concentrations between 0.002–0.005 M produced a marked depression in the amplitude of the auricles but had little effect on the rate, slight

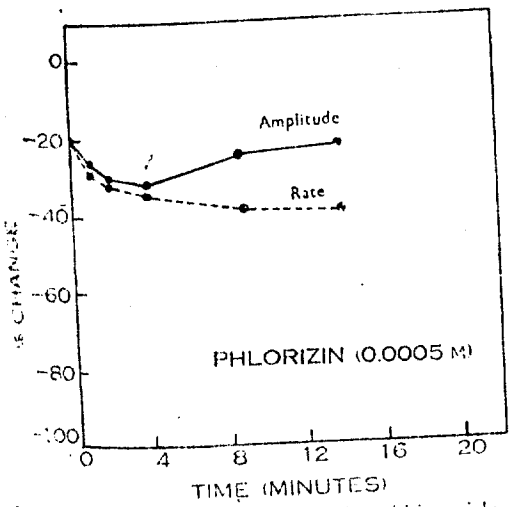


FIG. 11. The action of phlorizin on the rabbit auricle.

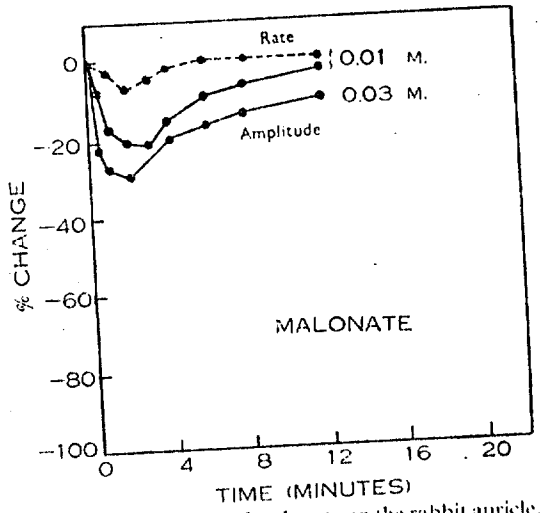


FIG. 12. The action of malonate on the rabbit auricle.

stimulation occasionally being the only effect observed. Since pyrophosphate combines with calcium and the medium became slightly turbid after its addition, the possibility was considered that the action upon the auricle was due to a decrease of ionized calcium in the medium. If calcium was added to the medium during pyrophosphate depression the amplitude returned to normal, but the increased rate was unaffected. Lack of calcium in the medium leads to depression of the amplitude, but the rate is never stimulated and is occasionally slightly reduced. Recovery of the auricles after low calcium was more rapid than after pyrophosphate depression. We may conclude, therefore, that the effects produced by pyrophosphate were partially due to a lack of ionized calcium, but that the pyrophosphate also had a definite action of its own. Since these actions could not be separated conveniently, the action of pyrophosphate was not studied farther and the curves of its action are not included in the Figures.

Malonate

The rabbit auricle was not strongly depressed by malonate: at high concentrations (0.02–0.03 M) a maximum inhibition of only 30 per cent in rate and amplitude was reached. Furthermore, the auricles spontaneously recovered in the presence of malonate at these high concentrations and returned to normal, or nearly normal, rates and amplitudes after 8–10 minutes. When the malonate was washed from the bath during the period of greatest depression, the recovery was very rapid, requiring only two to three minutes. Forssmann and Lindstén (1946) found that malonate in concentrations between 0.01–0.04 M depressed the ventricular contractions of the perfused rabbit heart but had little effect on the rate. It is remarkable that malonate at these concentrations should have so little depressant action on the auricles, for it is generally believed that malonate at a concentration of 0.005 M completely inhibits succinic dehydrogenase and therefore blocks the tricarboxylic acid cycle, a system upon which the cardiac muscle is thought to depend. Recently it has been reported that somewhat higher malonate concentrations, such as those used here, will prevent the oxidation of oxalacetate in homogenates, this action producing an additional derangement of the tricarboxylic acid cycle (Pardee and Potter, 1949). It seems unlikely that failure to penetrate the muscle cells could have been responsible for this lack of action, inasmuch as exposures of 20–30 minutes to malonate did not lead to any marked alteration of auricular function. The strange behaviour of cardiac cells under the influence of malonate was noted previously on rat heart slices (Webb, Saunders, and Thienes, 1949b). In this preparation malonate produced an initial depression of the respiration, but this was followed by a recovery to values near the normal rate. Furthermore, in the rat heart slices it was known that the succinic dehydrogenase was inhibited, for no rapid increase in oxygen uptake was observed when succinate was added as invariably happened when malonate was absent. Rat heart slices can respire adequately in the presence of high concentrations of malonate, and auricles can function quite normally under these conditions. We must conclude that cardiac tissue can respire and function when this particular step in the tricarboxylic acid cycle is blocked. It may well be that when this block is imposed in an organized cellular system, the cycle can be short-circuited; perhaps under such conditions oxalacetate may be formed directly from α-ketoglutarate, or pyruvate or its activated fragment be taken into the

cycl y combination with something other than oxalacetate. It will be shown late at the small amount of depression observed with malonate is probably not due he inhibition of succinic dehydrogenase.

Interactions of substrates and inhibitors on the auricles

eriments were performed to investigate the abilities of various substrates to c teract or antagonize the depressions produced by metabolic inhibitors. The ically, if an inhibitor acts specifically on one enzyme in a series of enzymic react s, the addition of the intermediate substrate that is normally formed by the tion of this enzyme should allow at least partial recovery of the functional activity of the tissue. If the inhibitor acts not only on this one enzyme, but upon other s well, the amount of recovery produced by the added intermediate will be roughly proportional to the specificity of the inhibitor effect. The possibility must always be considered that an added substrate, in the presence of an inhibitor, may initi energy-forming reactions that are not normally important ; thus, a stimulation the auricles by a substrate during an inhibitor depression does not necessarily mean at there is a direct relationship between the inhibitor and the substrate in the lar metabolism.

The relatively weak effect of malonate on the auricles was puzzling, and attempts were efore made to determine whether the depression that was produced was rela the commonly accepted action of malonate on succinic dehydrogenase. Ma e prevents the formation of fumarate from succinate, and recovery from mal e depression should, therefore, be observed after the addition of fumarate. If ma te acted simultaneously elsewhere in the tricarboxylic acid cycle this would not essarily be true. Three types of experiment were performed.

(1) In two experiments the effects of malonate (0.01–0.03 M) were first determin and these were compared with the effects of adding malonate and fumarate (0.001 0.01 M) together. No modification of the action by fumarate was observed. In a typical experiment the average maximum depression of amplitude produced by malonate alone was 25.7 per cent ; when both malonate and fumarate were added together the depression was 27.7 per cent. The changes in rate were likewise comparable.

(2) In the second type of experiment fumarate (0.001–0.01 M) was added to auricles already depressed by malonate (0.02 M). No stimulating effect was observed ; in fact, an additional depression was usually seen.

(3) In the third type of experiment the auricles were allowed to remain in contact with fumarate (0.005 M) for five minutes before the addition of the malonate (0.01 M) in order to ensure penetration of the fumarate. Again no change in the response to malonate was observed.

It therefore seems likely that the malonate depression is not due solely to inhibition of succinic dehydrogenase. It is more reasonable, I believe, to assume that malonate at such concentrations exerts a toxic action on the cell surface, a depression such as is seen with any of such substances under similar conditions. Acetate (0.001 M) and pyruvate (0.005 M) were also unable to counteract the depression produced by malonate (0.02 M), only additional depression being observed.

The depression produced by fluoride (0.005–0.02 M) was not counteracted by succinate (0.001 M), acetate (0.001 M), or citrate (0.001 M). However, pyruvate (0.005 M) showed some ability to stimulate the rate of the auricle in the presence of fluoride, but the amplitude was usually unaffected. This is in harmony with most of the results obtained with pyruvate, namely that it acts more particularly upon the mechanisms involved in rate than upon those involved in the amplitude of contraction. Complete antagonism of fluoride with pyruvate was never observed.

Iodoacetate, like fluoride, is generally supposed to act predominantly on the glycolytic stages of metabolism, inhibiting the formation of pyruvate from glycogen or glucose. However, there are reasons for supposing that in cardiac tissue the action is not specific. The heart muscle presumably obtains energy from both glycolytic and post-glycolytic phases of metabolism, although a relatively small amount from the former (approximately 15–20 per cent of the total). If iodoacetate acted only on glycolysis, the addition of pyruvate should restore the activity of the heart muscle to about 80 per cent of its normal value, only the energy from the glycolysis now being lost to the tissues. Three experiments were performed to test this.

(1) In the first experiment the concentration of iodoacetate was quite high (0.001 M), the auricles had been in contact with the inhibitor for 15 minutes, and the resulting depression was severe (rate – 40 per cent, amplitude – 95 per cent). Addition of pyruvate (0.01 M) gave no stimulation, the rate and amplitude continuing to decrease and contracture to develop. After the auricles were completely stopped at 30 minutes the addition of pyruvate was without effect.

(2) In the second experiment the concentration of iodoacetate was lower (0.0002 M), the time of exposure to the inhibitor was 25 minutes, and the auricles were very depressed (rate – 83 per cent, amplitude – 87 per cent). The addition of pyruvate (0.005 M) slightly stimulated the rate but had no effect on the amplitude. The normal rate before iodoacetate was added was 72, the iodoacetate had reduced it to 12; pyruvate increased the rate to 18 in two minutes and to 24 in four minutes. Later in the depression pyruvate had no effect at all.

(3) In the third experiment the iodoacetate (0.0002 M) was allowed to act for 15 minutes, the auricles being moderately depressed (rate –19 per cent, amplitude – 53 per cent). Pyruvate (0.005 M) slightly stimulated both rate and amplitude, the rate increasing by 15 per cent in two minutes and the amplitude increasing by 32 per cent within 30 seconds; these were the maximum effects and were only temporary, the depression of the auricles proceeding as if the pyruvate had not been present. Later in the depression pyruvate was added at both 0.001 M and 0.01 M without any effect.

One may conclude that pyruvate is only partially able to counteract the depression produced by iodoacetate, and that the higher the concentration of iodoacetate and the longer its action, the less recovery may be observed. These experiments confirm previous conclusions that iodoacetate is not a specific poison of the glycolytic phase of metabolism in mammalian cardiac muscle. Acetate (0.001–0.01 M) showed no ability to counteract the iodoacetate depression.

pression of the auricles produced by fluoroacetate was partially antagonized by ruvate. This was demonstrated in two experiments.

In the first experiment the concentration of fluoroacetate was 0.001 M, the tim f exposure was two hours, and the depression was very marked (rate −79 per ce mplitude −83 per cent). Pyruvate (0.01 M) increased the rate by 133 per ce d the amplitude by 18 per cent within one minute, the rate returning to half its mal level and the amplitude to only 20 per cent of its normal value. The ade on of pyruvate at the same concentration 35 minutes later produced even gre r stimulation, the rate increasing by 200 per cent and the amplitude by 126 per nt in two minutes.

In the second experiment the fluoroacetate (0.001 M) was allowed to act for on 25 minutes and the auricles were only moderately depressed (rate −24 per ce mplitude −25 per cent). Pyruvate (0.005 M) gave some stimulation, increasing rate by 8 per cent and the amplitude by 4 per cent within one minute. Af n exposure of 50 minutes, when the auricles were more depressed (rate −41 pe , amplitude −62 per cent), pyruvate at the same concentration stimulated m ccessfully, the rate increasing by 40 per cent and the amplitude by 21 per ce The effect on the amplitude was quite temporary and after three or four mi the auricles were again being progressively inhibited, but the rate effect re d for a much longer time.

Discussion

metabolic inhibitor used in the present investigation has been found to exe unique and characteristic action upon the auricle, the effects differing from on other with respect to (*a*) the concentration-action curves, (*b*) the time course of a on, (*c*) the relative actions on rate, amplitude of contraction, and muscular relaxation, (*d*) the ability of the cardiac muscle to er spontaneously in the presence of the inhibitor, and (*e*) the ability to recover after the inhibitor has been removed. Each inhibitor can be distinguished from the rest by its action on the rabbit auricle. The problem, however, is to determine whether any useful information on the mechanisms of cardiac function may be derived from such data in conjunction with the results on substrates, substrate depletion, and anoxia.

The auricles may be stopped in either diastole (state of relaxation) or systole (state of contraction). Substances which arrest the auricles rapidly always stop them in a state of relaxation. The process of contracture, by which the auricles stop in systole, requires time for its development and is not observed early in the action of any substance. There are substances which will rapidly arrest the auricles in diastole and, with continuing action, will induce contracture, the end result being systolic arrest. In the present experiments with rabbit auricles, the following have been found to lead to contracture: iodoacetate, fluoroacetate, fluoride, cyanide, anoxia, and substrate depletion. All of these would be expected to produce a state of reduced metabolic activity with a decrease in the amount of energy available to the muscle. This inhibition of the flow of energy would result in a gradual loss of ATP (and other substances containing high-energy phosphate bonds), such having been observed in the past under similar conditions. Simultaneously with the depletion of ATP the cardiac muscle becomes less and less able to relax, and when the ATP level becomes sufficiently low the muscle goes into complete contracture and

will beat no longer. The ATP concentration need not drop to zero for this to occur. This conception is in harmony with the views of Szent-Györgyi (1947) on skeletal muscle, ATP being required for the relaxation of the muscle fibrils as well as for their contractile activity. The auricular muscle is in a low-energy state when it is contracted and in a high-energy state when it is relaxed. Adding glucose or pyruvate to a muscle in contracture would provide some ATP and the muscle would be able to relax and beat again.

Let us consider auricles that have been stopped in the relaxed state of diastole by some metabolic inhibitor. Why does the muscle not contract? There is certainly present a sufficient concentration of ATP. There is nothing fundamentally wrong with the contractile mechanisms, for the muscle can be made to contract by electrical stimulation, and it will recover from such contractions for a period of time. The initiating reaction for contraction must be depressed. The functioning of cardiac muscle involves not only the mechanisms of contraction and relaxation, but also processes that are rhythmic in nature and serve to stimulate these contractions. Normal contraction is induced by a wave of depolarization (accompanied simultaneously by other changes) which sweeps over the muscle and in some manner causes the actomyosin to be transformed into its low-energy state. This stimulating process can be roughly divided into three phases: the discharge of the impulse from the pacemaker cells, the conduction of this impulse, and the excitatory reaction by which the actomyosin is affected by the impulse. These process are all dependent on metabolic reactions for their energy. Maintenance and restoration of the cellular membrane potentials require energy, and further energy may be required for the synthesis and distribution of substances involved in these processes. It is likely that these excitatory mechanisms are inhibited in auricles that have been arrested in diastole. Occasionally a substance will stop the heart by depressing the discharge of impulses from the pacemaker cells, but usually it would seem to be a matter of depression of conduction or of the excitatory reaction (the process by which the actomyosin is stimulated by the conducted impulse will be here termed the "excitatory reaction"). In many cases the amplitude of the auricles was gradually depressed until arrest occurred; immediately before arrest the rate was depressed relatively little. Observation of such auricles leads one to the conclusion that the pacemaker cells are still discharging after the auricles have ceased to beat, the failure being due to disturbances in conduction or the excitatory reaction. If such is the case, it lends support to the view that partial inhibition of the amplitude is due to the failure of a certain fraction of the contractile units to react, the contraction of each unit being all-or-none in nature. Progressive inhibition of the excitatory reaction would lead to a gradual decrease in the number of units responding to the conducted impulse, and this in turn would lead to a progressive inhibition of the amplitude of contraction. Conduction and the excitatory reaction may be more sensitive than the processes of contraction and relaxation to metabolic inhibitors and anoxia because they do not possess a store of ATP, most of this substance being found in the actomyosin or in the enzymes involved in phosphate transfer.

When the amplitude of contraction of cardiac tissue is reduced to some fraction of its normal value (no change in the degree of relaxation having taken place), we may interpret this in two general ways. Either there is a smaller fraction of

all-none units contracting, or each unit in the muscle is contracting proportionately less. The solution to this problem is fundamental to cardiac physiology and pharmacology. Does cyanide, for example, when it inhibits the auricular amplitude by [illegible] per cent, prevent completely the contractions of 20 per cent of the contractile units, the remaining units contracting normally, or does it reduce the contraction of each unit an average of 20 per cent? This is not merely the old question of whether the concentration-action curve is an expression of the action of a substance on cellular processes or whether it is derived from the distribution of susceptibilities of the individual cells. A depressant may inhibit the excitatory reaction either by raising the threshold of response of the contractile units or by reducing the excitatory state that is initiated by the passage of the impulse. In the former case we should be measuring a distribution of susceptibilities, but in the latter case it would be a matter of a true action on a cellular process. There is no *a priori* reason why both actions may not be of importance, depending on the substance that is producing the depression. It should also be pointed out that, although the contractile units may normally behave in an all-or-none manner, it is entirely possible that various conditions may modify the degree of contraction.

How must we interpret the changes in amplitude brought about by the action of the metabolically important substances on the rabbit auricle? The results of the present investigation can be best explained by assuming that these substances modify the excitatory reaction and not the contractile mechanisms directly. Only when the cardiac muscle begins to go into contracture is the contractile mechanism itself altered. If this is the correct interpretation, it would seem that our conceptions of the actions of substances on cardiac muscle must be modified. Such an interpretation would also apply to the actions of drugs on the heart.

Throughout this investigation it was found that the changes in rate and amplitude of the auricles were relatively independent of each other. An increase or decrease in the amplitude had no particular effect on the rate, nor did changes in the rate, within limits, affect the amplitude. Quite frequently one would remain constant during a marked change in the other, and occasionally they would change in the opposite directions. The fundamental energy-forming enzymatic reactions may be quite similar in the processes that control rate and amplitude, but the paths of energy flow and utilization are different. It is true that most substances change the rate and amplitude in the same direction. This is perhaps more readily understandable when we consider changes in amplitude as arising, not from alterations in the contractile mechanisms, but from modifications of some cellular surface phenomenon that is involved in the excitatory reaction, such a surface state being related to the cellular membrane changes occurring in the pacemaker cells. In whatever way we consider the cyclical discharge of impulses, we must assume that there is the development of a depolarized state and that this is propagated over the cardiac muscle, this being responsible, directly or indirectly, for the excitatory reaction and the contraction of the myofibrils that lie in the region over which the impulse is passing. We can thus see a possible connexion between rate and amplitude, a relationship that implies no necessary simultaneous behaviour, but which, in the majority of cases, will lead to parallel

behaviour with respect to direction of change. However, the magnitudes of the changes in rate and amplitude need bear no relationship to one another.

Development of contracture was always a late process, following earlier alterations in the auricular rate and amplitude. We have supposed that this was due to a greater reserve of ATP or other substances containing high-energy bonds. It is indeed possible that the membrane phenomena of impulse discharge, conduction, and the excitatory reaction do not utilize ATP or similar substances, but that the cyclical processes involved are more directly dependent on the oxidative sequence of enzymatic reactions. Most of the metabolic inhibitors investigated, as well as substrate depletion and anoxia, depressed the amplitude more than the rate. This might be explained by assuming that the rate processes require less energy than those involved in the excitatory reaction, but this can hardly be the entire answer since there are instances in which the rate is depressed as much as the amplitude. The metabolic systems involved in the rate and amplitude may also be assumed to be somewhat different, and this is quite likely from a consideration of the characteristic actions of the various inhibitors, but it would explain only the differences in behaviour and not the basic fact that the rate is more resistant to change than the amplitude. Another explanation might be that the rate and amplitude mechanisms alter their functional level to different degrees in response to an equivalent change in some common factor. For example, if we assume that both the rate of discharge and the effectiveness of the impulse on the contractile units depend on changes in the degree of electrical polarization of the cell membrane, it is conceivable that a depression of the resting potential or a depression of the rate of repolarization would inhibit both processes. However, owing to the particular circumstances, the number of units reacting would be depressed more readily than the discharge rate. The excitatory reaction is probably quite delicately adjusted to the changes occurring at or near the cell surface. This delicate balance assures a ready adaptability of the cardiac muscle.

We might expect, for the same reasons, that glucose and pyruvate would stimulate the amplitude more than the rate in substrate depleted auricles. This was found to be true for glucose, but pyruvate acted more particularly on the rate. Quantitative comparisons are complicated by the third action of these substrates, the stimulation of relaxation, an action somewhat more readily produced by glucose than by pyruvate. However, it was clear that pyruvate, both in substrate depleted auricles and in those poisoned by inhibitors, stimulated the rate more effectively than the amplitude and more effectively than it produced relaxation of the muscle. From this it might be concluded that the oxidations in the tricarboxylic acid cycle are the main source of energy for the rate processes. Supplying glucose does not lead to a sufficiently rapid formation of pyruvate, and therefore giving pyruvate directly has a greater stimulating effect. Why, however, is pyruvate less effective than glucose in stimulating the amplitude? The energy derived from the breakdown of glucose to pyruvate must be of greater importance to the processes involved in the contraction and relaxation of the muscle than it is to the rate processes. It is doubtful whether we are measuring the effect of either substrate on the excitatory reaction under such circumstances, the stimulation of ATP formation being the primary action in increasing activity of contraction. According to Szent-Györgyi (1947) the actomyosin requires so much ATP

be active at all, aside from the ATP that is utilized for relaxation. Since ATP can be formed from energy derived from the breakdown of glucose to pyruvate it is not so surprising that glucose is somewhat more effective than pyruvate in this respect. However, in the cellular membrane processes of impulse discharge and the excitatory reaction the conditions are different; as we have seen, it is possible that these mechanisms do not require ATP and may operate directly from the oxidative systems, especially the tricarboxylic acid cycle. Since the tricarboxylic acid cycle can continue to operate in the metabolism of fat, the observation that the rate is more steady during inhibition of glycolysis (iodoacetate) than it is during inhibition of the tricarboxylic acid cycle (fluoroacetate) is explained. There remain, however, a number of problems—for example, the anomalous behaviours of malonate and fluoride—and these ideas are presented only as a working hypothesis.

The metabolic activities of contracted and relaxed cardiac muscle may be different. The metabolism may occur in cycles, in phase with contraction and relaxation, so that the conception of the metabolism as a steady state would be erroneous. Deductions based on steady state conditions would not necessarily be applicable, nor would arguments based on limiting reactions always be valid. This must be kept in mind in considerations of the action of metabolic substrates and inhibitors on the heart. Determinations of the ability of heart slices to oxidize various substrates may give information on only one phase of the cycle; it is likely that such slices are in a state of contracture. The same reasoning will be applicable to the actions of inhibitors. Substrates that are well oxidized by a contracted muscle may produce no stimulation of the normally beating auricle because their oxidation occurs at phases of the cycle where their energy cannot be utilized; only when the cardiac muscle has become abnormal, when these cyclical processes have become distorted, or when the muscle has gone into contracture will these substrates be oxidized with the production of utilizable energy. Similar metabolic cycles of activity probably occur in the pacemaker cells during rhythmic discharge of impulses and near the surfaces of the muscle cells as the propagated impulses pass.

Furthermore, one is not justified in assuming that there is a general synthesis and supply of ATP upon which all the processes in the cardiac cell must draw. ATP is probably used in only certain functional mechanisms and is synthesized in these particular regions. The concentration of ATP as determined by analysis of the entire muscle can seldom be correlated with the functional activity of the tissue for this reason. When one considers the various processes which control the beat of cardiac tissue, the different metabolic requirements of these processes, the spatial relationships, and the cyclical nature of the metabolism, it is easy to understand why it is so difficult to correlate analyses for substances containing high-energy bonds and for other metabolic intermediates with the level of activity of the cardiac muscle.

Summary

1. The actions of thirteen metabolic substrates and intermediates on the rabbit auricle have been investigated, and it was found that they all depress the normal auricle, with regard to both rate and amplitude, except citrate which at higher concentrations stimulated the rate.

2. The behaviour of the beating auricles during depletion of substrate was observed and the abilities of various substrates to revive the auricles have been determined. It was found that only glucose and pyruvate produced marked effects; glucose produced a more marked effect on muscular relaxation, and pyruvate acted particularly upon the rate.

3. The actions of eight metabolic inhibitors on the rabbit auricle have been investigated and each inhibitor was found to produce a characteristic effect. The actions of these inhibitors on the functioning of the auricles does not always parallel their commonly accepted actions on enzymatic systems, and it is concluded that unknown pathways of metabolism exist in cardiac muscle.

4. The abilities of substrates to revive auricles that have been depressed by inhibitors was studied, and the bearing of these results on the specificity of inhibitor action and the nature of myocardial enzyme systems are discussed.

5. None of the observed actions of metabolic substrates and inhibitors are due to an action on the acetylcholine equilibrium in the auricular muscle, because the presence of atropine did not modify them.

6. The mechanisms by which the auricles can be stopped in diastole or systole are discussed in the light of the information obtained on the actions of substrates and inhibitors.

7. It is questioned whether changes in amplitude are directly related to contractile mechanisms. The results obtained could be best explained by assuming that the actions of the substances investigated were upon the mechanisms by which the propagated impulse stimulates the actomyosin to contract into its low-energy state. This important phase of the cardiac functioning is termed the "excitatory reaction". Depression of this excitatory reaction, either by reducing the excitatory state of the cellular membrane or by increasing the stimulation threshold of the contractile units, leads to a reduced number of units contracting and consequently to a reduced amplitude.

8. Changes in rate, amplitude, and degree of relaxation were shown to be relatively independent of each other. From the actions of substrates and inhibitors as well as substrate depletion and anoxia, on the auricles, an attempt was made to correlate certain phases of metabolism with the mechanisms involved in rate, amplitude, and relaxation. It was concluded that contraction and relaxation are dependent on a certain supply of ATP but that the rhythmic discharge of impulses and the excitatory reaction are more directly dependent on the oxidative reactions of metabolism, particularly the tricarboxylic acid cycle.

9. Some general problems in the correlation of cardiac metabolism with cardiac function are discussed.

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Effects of Fatty Acid Anions on the Electroencephalogram of Unanesthetized Rabbits

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ABSTRACT

The i.v. injection of 4 mM/kg of buffered fatty acid anions of propionate, butyrate, valerate and caproate evoked electroencephalographic (slow high amplitude waves) and behavioral signs of sleep. The long chain fatty acid compounds were more effective than the short. On the other hand, comparable solutions of glucose, NaCl, lactate, acetone and Beta-hydroxybutyrate had no such effect. The evidence indicates that the fatty acid anion effect is mediated through the central nervous system and is not a secondary result of changes in cardiovascular physiology. CNS stimulation with amphetamine sulfate does not alter the depressant effect of the fatty acid anions.

THE SHORT CHAIN fatty acids, aside from their serving as metabolic substrates, are known to have a large variety of biological effects such as bactericidal (1), fungicidal (2) and hemolytic (3) action. That they also have a narcotic action and when given in sufficient amounts produce coma has been recently reported (4). Such behavioral changes, however, are not always correlated with changes in cortical potentials (5–8). Thus it seemed of interest to observe the electroencephalographic as well as other physiological alterations which may attend the administration of these compounds in order to gain a better understanding of the mechanism of action of the fatty acid induced narcosis. In this study EEG and other physiological changes observed in unanesthetized rabbits following the injection of neutralized short chain fatty acids (pH 7.4) are presented.

METHODS

Twenty-five unanesthetized adult male albino rabbits weighing approximately 2 kg were used for these experiments. The legs were tied securely to an animal board but the head was free to move. The conscious animal holds the head erect in this position whereas the head drops onto the animal board when unconsciousness results from the administration of a depressant drug.

The cortical electrical activity was recorded with a four-channel, ink-writing Grass III D electroencephalographic instrument. Three steel electrodes were implanted into the cranium over the right and left limbic and left motor cortex. The electrodes were located 2 mm anterior to the transverse suture and 2 mm lateral to the longitudinal, for the motor area; and 4 mm posterior to the transverse suture and 2.5 mm lateral to the longitudinal suture, for the limbic area. A local anesthetic (0.5% pontocain HCl, Sterling-Winthrop Research Institute) was administered subcutaneously over the dorsum of the cranium and the skin laid open prior to the electrode implantation.

The solutions of the fatty acid anions were freshly prepared by adding a known amount of the fatty acid to distilled water and then neutralizing with NaOH to a pH of 7.4. The final concentration of the fatty acid anion was 1 M. Only clear homogenous solutions were used. These solutions are not hemolytic in the amounts given. The fatty acids studied were propionic, butyric, valeric and caproic. Also tested were 1-M solutions of lactate, acetone, Beta-hydroxybutyrate, NaCl and 50% glucose. In a typical experiment 4 mM/kg body weight were injected into the marginal ear vein.

One channel of the EEG instrument was used to record simultaneously the ECG of each animal. Needles were inserted subcutaneously into the left hind leg and right foreleg for this purpose. Arterial blood pressure was obtained by means of a mercury manometer connected to the femoral artery by a cannula filled with a heparin solution.

Approximately 25 ml of a 50% glucose solution were injected either prior, simultaneously or subsequent to the administration of fatty acid anion in six animals in order to determine whether these procedures would alter the effect of the fatty acid injection. In addition, 25 mg of Amphetamine sulfate was administered intraperitoneally in two animals in order to ascertain whether the 'alerting' of the EEG by this agent would alter the effects observed by the administration of fatty acid anion.

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RESULTS

The intravenous injection of propionate, butyrate, valerate and caproate immediately induced EEG changes from a resting type to one of sleep. The wave frequency diminished and the amplitude increased as can be seen in the record shown in figure 1. Furthermore, 'sleep spindles' (14 cps) of the kind described by Rinaldi and Himwich (8) were frequently observed to be superimposed on the sleep pattern. During these EEG alterations overt signs of drowsiness or sleep always occurred and the typical 'alert' EEG responses (i.e., low-voltage fast activity) to nociceptive and auditory stimuli were diminished or absent.

Although the depth and duration of sleep induced by these substances varied considerably from animal to animal the over-all pattern was the same. The EEG changes persisted from 40 seconds to about 45 minutes, depending to a great extent upon the chain length of the compound injected. Thus, the longer carbon chain fatty acids caproate and valerate were more effective than butyrate or propionate. Recovery from these injections were usually abrupt, the head was elevated rapidly and thereafter the EEG record returned to a control pattern.

In contrast to the injection of fatty acid anions, similar solutions of lactate, acetone, Beta-hydroxybutyrate, NaCl and glucose did not alter the resting EEG pattern nor the overt appearance of the animal. One such negative result obtained from an acetone in-

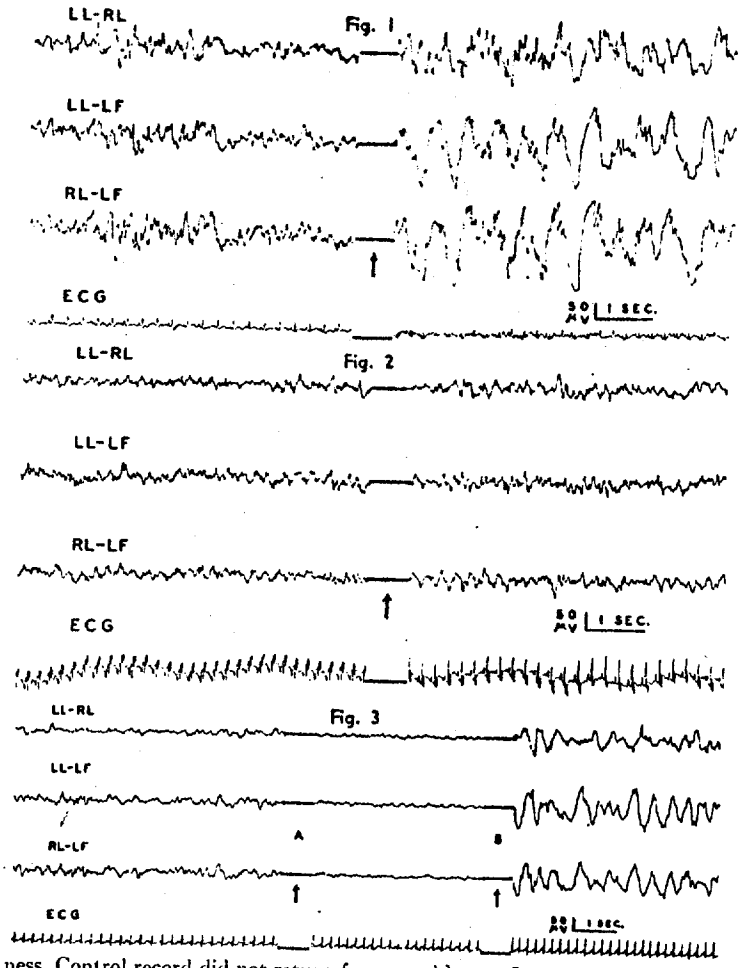

FIG. 1. Cerebral electrical activity before and after administration of 4 mM/kg of butyrate in an unanesthetized rabbit. Electrographic wave frequency diminished and amplitude increased as a result of butyrate injection (indicated by arrow). This EEG sleep pattern was accompanied by behavioral signs of sleep, which were induced immediately with the injection. Leads from above downward are as follows: *1*) left limbic, right limbic (*LL-RL*); *2*) left limbic, left frontal (*LL-LF*); *3*) right limbic, left frontal (*RL-LF*); *4*) electrocardiogram (ECG).

FIG. 2. Cerebral electrical activity before and after administration of 4 mM/kg of acetone in an unanesthetized rabbit. Electrical brain wave pattern is essentially the same as control record (left of *arrow*) and no overt signs of depression were observed. Leads as in fig. 1.

FIG. 3. Complete failure of an Amphetamine sulfate induced electrographic alerting response (between *arrows* at *A* and *B*) to prevent appearance of an EEG sleep pattern evoked by butyrate (right of *arrow* at *B*). Control record is shown at left. Butyrate solution was injected 16 min. following intraperitoneal administration of 25 mg of Amphetamine sulfate. It should be noted that 40 min. after butyrate injection sleep EEG pattern was replaced by one of alertness. Control record did not return for several hours. Leads as in fig. 1.

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FIG. 1. Cerebral electrical activity before and after administration of 4 mM/kg of butyrate in an unanesthetized rabbit. Electrographic wave frequency diminished and amplitude increased as a result of butyrate injection (indicated by arrow). This EEG sleep pattern was accompanied by behavioral signs of sleep, which were induced immediately with the injection. Leads from above downward are as follows: *1*) left limbic, right limbic (*LL-RL*); *2*) left limbic, left frontal (*LL-LF*); *3*) right limbic, left frontal (*RL-LF*) electrocardiogram (ECG).

2. Cerebral electrical activity before and after administration of 4 mM/kg of acetone in an unanesthetized rabbit. Electrical brain wave pattern is essentially the same as control record (left of *arrow*) and no overt signs of depression were observed. Leads as in fig. 1.

FIG. 3. Complete failure of an Amphetamine sulfate induced electrographic alerting response (between *arrows* at *A* and *B*) to prevent appearance of an EEG sleep pattern evoked by butyrate (right of *arrow* at *B*). Control record is shown at left. Butyrate solution was injected 16 min. following intraperitoneal administration of 25 mg of Amphetamine sulfate. It should be noted that 40 min. after butyrate injection sleep EEG pattern was replaced by one of alert-

jection is shown in figure 2. Even several repeated injections of acetone failed to elicit signs of depression. A subsequent injection of butyrate, however, proved these same animals to be sensitive to the fatty acid anion. Occasionally an 'alerting' pattern appeared on the EEG as an after effect of the needle injection, but this usually disappeared within 20 seconds.

Although the injection of fatty acid anion usually caused a drop in blood pressure of 11–17 mm Hg, such alterations did not correspond with the sleep pattern observed. The onset and duration of this decrement varied with each animal, in one instance the pressure decreased after an alteration in the EEG was observed. Moreover, the blood pressure did not fall below 70 mm Hg nor was the drop very rapid; two conditions which are known to produce abnormal brain wave patterns (8). Usually the pressure decreased slowly, reaching a low point in from 1–5 minutes and then rising again to initial value while the signs of sleep in the animal continued.

Similarly, there were great individual variations in the heart rate, and changes in the character of the ECG upon the administration of the fatty acid anions. Often an initial transient bradycardia, with a subsequent cardiac acceleration, and changes in the ECG complex were recorded. These same alterations were frequently associated with the injection of lactate, acetone, NaCl and glucose solutions; substances which did not produce a sleep condition. Occasionally neither of these variations were evident with the injection of fatty acid anion and, therefore, such changes were not peculiar to the production of the sleep pattern.

The injection of glucose either prior, simultaneously, or subsequent to the fatty acid injection did not alter the effects of fatty acid injections. Furthermore, the intraperitoneal injection of 25 mg of Amphetamine sulfate which established an 'alert' EEG pattern prior to the administration of fatty acid solutions did not prevent the usual depressant effects of the fatty acid anion (fig. 3).

DISCUSSION

It is clear that the fatty acid anions induce high-voltage slow wave and 14 cps spindles in the EEG which may be termed a 'sleeping' pattern. Furthermore, concomitant overt signs of mild or deep sleep appeared as a result of the injection of these anions. This is important since a so-called sleep type of EEG is not always associated with sleep of the animal (5–8). It has been recently reported (4) that the fatty acid anions have a narcotic action in a wide variety of animals and therefore this phenomenon is not peculiar to the rabbit. In this regard it should be noted that the EEG pattern induced by fatty acid anions in the rabbit is similar to that observed under deep sodium pentobarbital anesthesia (9) or chlorpromazine medication (10).

The sleep provoked in these animals does not seem to be related to changes in blood pressure since these changes are too small, slow and transitory to account for this reaction. In addition, similar changes in heart rate and blood pressure were also often observed with the experimental substances which did not induce sleep. Occasionally none of these alterations were evident following the injection of fatty acid solutions. The finding that the longer chained fatty acid anions in general produced more profound signs of sleep than did the shorter ones is in agreement with previous investigators (4).

Since the fatty acid anions were effective in provoking the sleep pattern whereas ketone substances of the same molar concentration were not, it seems reasonable to suggest that the fatty acid anion may be important in the pathogenesis of diabetic coma. The high concentration of lipids in the blood during severe diabetes in human beings supports this belief. The possibility that these compounds may account for insulin resistance in diabetic acidosis has been suggested (11). Acceptance of such an hypothesis would, however, depend upon more evidence relating the onset of diabetic acidosis to fatty acid blood levels. The sudden onset of effects mediated by fatty acid injections indicates that their action is a direct one.

It has also been suggested that the histotoxic phenomena observed in diabetic coma may be related to the effects of ketone substances (12, 13). Indeed, the injection of sufficient quantities of these compounds will induce coma in animals (13–15) but there is

no good correlation between blood ketone levels, the onset of coma (16-18) and the clinical recovery from diabetic acidosis during therapy (19, 20). Moreover, Fisher (15) recently showed that hyperketonema could be produced in rabbits and maintained for several hours in a range comparable to that observed in human diabetic acidosis without apparent toxic effect. This author also observed 11 patients with arterial ketone values which ranged from 34.9-123.0 mg % whose mental state was normal. He concluded from his studies and a review of the literature that it was unlikely that the ketone bodies are the primary cause of the clinical picture of diabetic coma and that some other potent agent is the dominant factor.

The fatty acid anion action is evidently not one of competitive inhibition of glucose metabolism *in vivo* since injection of large amounts of this substance did not alter the depressant effects of fatty acid solutions. Nor is this depressant action prevented by the administration of the CNS stimulant Amphetamine sulfate. The absence of effect with compounds which have a hydroxyl group in the hydrocarbon part of the molecule (i.e., lactate and Beta-hydroxybutyrate) as well as a more noticeable depression with increases in carbon chain length, and the relatively quick return to the resting type of EEG are properties similar with those shown in the inhibition of metabolic reactions in vitro (11). This would indicate that the mechanism of action at the chemical level may be the same. In the case of the inhibitory action on metabolic reactions, the negatively charged fatty acid anion is believed to bind to some positively charged site on enzymatic protein adjacent to nonpolar amino acid residues and thus interfere with the normal formation of the enzyme-substrate complex (11). It is possible therefore that some enzymatic reaction necessary for consciousness is inhibited by the fatty acids.

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Depression of feed intake of goats by metabolites injected during meals

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BAILE, CLIFTON A., AND JEAN MAYER. *Depression of feed intake of goats by metabolites injected during meals.* Am. J. Physiol. 217(6): 1830–1836. 1969.—To test the relative depression of feed intake by various metabolites, goats were maintained in stalls with feeders equipped with photoelectric relays to activate pumps and recorders during spontaneous meals. Sodium acetate, propionate, butyrate, a mixture of the three salts (VFA) (pH = 6.5), and propionic acid were injected intraruminally at rates of 2.5, 5, 10, or 20 mmoles/min during eating. All solutions depressed feed intake although butyrate was least effective. Regression equations expressing the relationship between per cent depression of feed intake relative to control periods (Y) and average mmoles of salt injected per meal (X) were: for acetate—$Y = .39X - 5.18$, $r = .76$, $P < .01$; for propionate—$Y = .36X - .27$, $r = .69$, $P < .01$; for butyrate—$Y = .17X + 11.40$, $r = .50$, $P < .02$; and for VFA—$Y = .38X + 8.74$, $r = .79$, $P < .01$. Intraruminal injection of sodium lactate (pH = 6.5) was strong but variable while sodium formate (pH = 6.5) was relatively weak as a depressant. Glucose, $NaHCO_3$, or mixtures of the two caused only small depressions. Because volatile fatty acids are the main products of fermentation in the rumen and because their production rates increase with [illegible]ing, the feeding-depressant effects of acetate and propionate [illegible] be important in regulation of energy balance in ruminants especially when grain rations are fed.

feedbacks for control of feed intake; feed intake control; ruminants; volatile fatty acids

EXPERIMENTS WITH RUMINANTS, e.g., goats and sheep, have shown that they control their feed intake to maintain energy balance under many conditions (1, 7, 16, 17, 19, 23), but not when fed some roughage rations (14, 15). Lesions in the ventromedial area of the hypothalamus result in hyperphagia (8), and lesions in the lateral areas of the hypothalamus cause aphagia and adipsia (3). Although [illegible]uminants are similar to monogastric animals in these aspects, the feedbacks and receptor sites for the control of feeding may be different. Under our laboratory and feeding conditions, neither hypothalamic temperature nor heat loss changes (6), glucose-utilization rate (4), nor ruminoreticular distension (9) is an apparently important feedback. Volatile fatty acids may act as important feedbacks in the control of feed intake in ruminants (1). They are produced by microbial fermentation in the ruminoreticulum, a large proportion are absorbed directly from the rumen, and they are the most important energy source for ruminants.

The present experiments were designed to characterize m[illegible] specifically the depression of feed intake of several intermediary metabolites of the rumen. A system was used so that goats fed ad libitum could be injected intraruminally with test solutions during spontaneous meals. Definite differences in feed-intake depression by the metabolites were shown under the conditions tested.

MATERIALS AND METHODS

Six goats (30–40 kg) were used in these studies, each prepared surgically with a ruminal fistula and fitted with a cannula of hard rubber. The goats were housed in a room with constant lighting and maintained at 24 ± 1 C. They were fed a concentrated diet ad libitum (less than 9% fiber, Omelene, Ralston Purina). The feed and water were measured and the orts determined between 8:00 and 9:00 AM each day. Also, at this time, the treatments were initiated, and the amounts of test solutions injected intraruminally during the preceding 24 hr were determined. The goats were locked in stanchions with an apparatus in front of them that monitored their feeding behavior (5). When a goat ate from its feed container, a light beam impinging on a photocell was broken which activated a relay that started a pump; thus the goats were injected intraruminally during spontaneous meals.

In all experiments the duration of each treatment was 2 days preceded by a 2-day control period and followed by a 2-day postinjection period during which no injections were given. Except in the case of some of the treatments of *experiment II*, all treatments of each experiment were replicated on each animal. The intraruminal injection rate of all solutions tested was 5 ml/min.

Experiment I. Goats 4, 5, and *6,* during the 2-day injection period, were given 2.5, 5, 10, or 20 mmoles/min of sodium acetate (pH = 6.5, Fisher NF grade) while eating. The concentration of acetate used was 0.5, 1.0, 2.0, or 4.0 M.

Experiment II. The treatments tested were 2.5, 5, 10, or 20 mmoles/min of propionic acid (Fisher certified grade) or sodium propionate (Fisher purified) (pH = 6.5). The 10- and 20-mmoles/min treatments of the acid and the salt were not replicated, whereas the other two treatments were replicated. *Goats 1, 2,* and *3* were used.

Experiment III. In this experiment, the same levels of injection as those of *experiment I* were given, but solutions of sodium butyrate (pH = 6.5, Fisher reagent grade) were used. *Goats 1, 2,* and *3* were tested.

Experiment IV. A mixture of the sodium salts (pH = 6.5) of acetate (55%), propionate (30%), and butyrate (15%) was injected intraruminally so that during spontaneous

meals either 2.5, 5, 10, or 20 mmoles/min were given on treatment days. The proportions of the salts were similar to those measured in the rumen fluid on control days. *Goats 2* and *3* were tested.

Experiment V. Sodium formate (pH = 6.5, Fisher certified) was injected at 10 mmoles/min in *goats 1, 2,* and *3.* Sodium lactate (pH = 6.5, Fisher) was injected intraruminally at 5 and 10 mmoles/min in *goats 3, 4,* and *6.* Treatments were replicated on each of the goats.

Experiment VI. Goats 4, 5, and *6* were given, on treatment days, an intraruminal injection of *1)* 6.7 mmoles/min of glucose, *2)* 6.7 mmoles/min of glucose plus 5 mmoles/min sodium bicarbonate (Fisher certified), or *3)* 5 mmoles/min sodium bicarbonate. The glucose provided the carbon equivalents of the 20-mmoles acetate treatment of *experiment I.*

A meal was defined as having at least 5 min duration, and at least 30 min must have elapsed since a previous meal. The goats broke the light beam across the feeders only infrequently between the so-defined meals, and such occurrences were disregarded in estimating meal pattern. The length of the meal was measured from the recorder charts and included the intermittent time the goat may have had its head out of the feeding container during a meal.

During experiments, rumen fluid samples were taken both on control and on treatment days. Samples were obtained prior to the feeding of fresh feed to goats in the morning. Samples were placed in a test tube containing $HgCl_2$, the pH was determined, and the samples were frozen. Samples were later prepared for volatile fatty acid analysis by gas-liquid chromatography (Hewlett-Packard dual column gas chromatograph, series 402) by the method of Baumgardt (12). In the preparation of each sample, a known amount of acetate-^{14}C (New England Nuclear Corp.) was added prior to the extraction procedure. Although the labeled acetate contributed an insignificant amount of acetate to the sample, the ^{14}C activity was determined and a recovery efficiency calculated. The acetate-^{14}C activity was counted in a scintillation system (11) (702 series scintillation counter, Nuclear-Chicago).

Feed and water intakes, meal frequencies, and meal lengths were tested for significance by paired *t* tests (control-period data were paired with the treatment-period data of each animal). The rumen fluid pH, total concentration of volatile fatty acids, and component proportions of the volatile fatty acids were tested for differences by using unpaired *t* tests of control and treatment data.

RESULTS

General. All data are presented as 24-hr means of 2-day preinjection, injection, and postinjection periods. The intraruminal injection of sodium salts resulted in an increased oral water intake by the goats; glucose or propionic acid solution did not cause an increase in water intake. Length and number of meals (as defined in the methods section) tended to be shorter during the higher injection rates of the test solutions when feed intake was depressed appreciably (Figs. 1–6).

Feed intake. Acetate, propionate, butyrate, a mixture of the three salts, and propionic acid caused greater depression

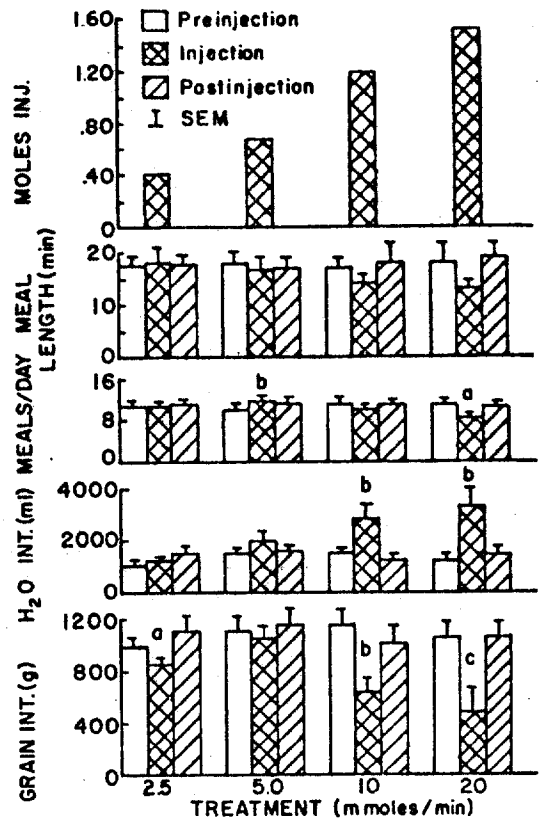

FIG. 1. *Experiment I.* Na acetate (pH = 6.5) was injected intraruminally into goats during spontaneous meals. Data are all presented as daily means of 2-day periods. Letters a, b, and c denote levels of significance .10, .05, and .01, respectively, of the difference between the preinjection and injection means as tested by paired *t* tests.

of feed intake with increasing rates of injection with one exception (Figs. 1–4). We have no explanation other than chance for the apparent trend toward a greater feed intake with injections of 5 mmoles than with 2.5 mmoles/min of acetate. The 5-mmoles/min treatment had the following effects: water intake was slightly increased and the rumen pH was increased ($P < .05$), Table 1; concentration of volatile fatty acids of the rumen fluid and the number of meals were increased ($P < .05$), and although the proportion of acetate in the rumen fluid did not increase ($P > .10$), volatile fatty acid concentrations did.

There was no difference between the effect on feed intake of sodium propionate (pH = 6.5) and propionic acid ($P > .2$), tested by a paired *t* test. Sodium propionate injections caused an increased oral water intake with increased rates of Na^+ injection whereas propionic acid injections caused reduced oral water intake with increasing rates of injection (Fig. 2).

Although the depression of feed intake by butyrate (Fig. 3) was somewhat less than that observed with acetate or propionate, the estimated digestible energy intake was not nearly so much depressed as by the other salts. The highest injection rate added an average of 570 kcal of digestible energy to the goats' energy intake per day.

Figure 5 shows the effects of sodium formate and sodium lactate. Feed intake was depressed only about 20% ($P > .10$) when an average of 0.70 moles was injected per day at 10

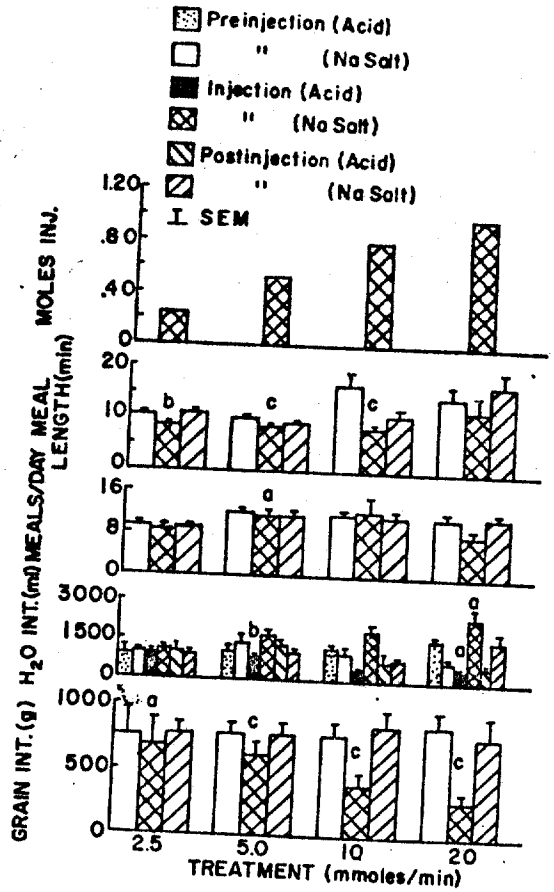

FIG. 2. *Experiment II.* Na propionate (pH = 6.5) or propionic acid was injected intraruminally during meals of goats fed ad libitum. Effect of acid and salt differed only in water-intake response and therefore data for other parameters are combined. All data are daily means of 2-day periods. Denotations of significance are same as in Fig. 1.

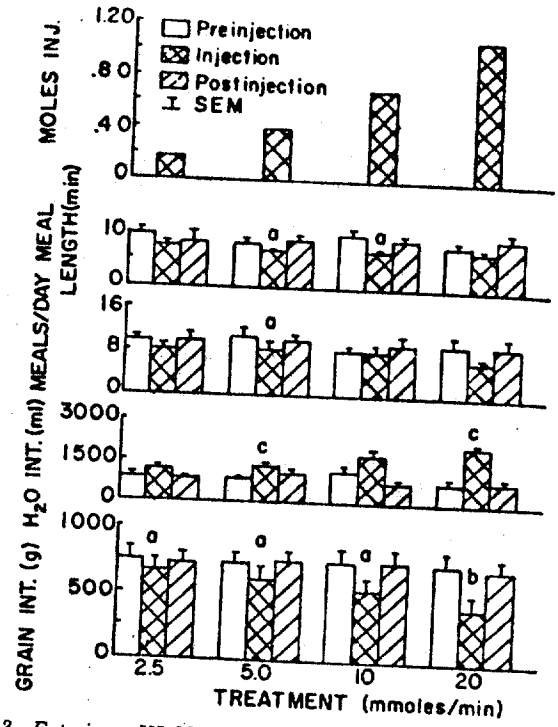

FIG. 3. *Experiment III.* Na butyrate (pH = 6.5) was injected intraruminally during spontaneous meals in goats. All data are daily means of 2-day periods. Denotations of significance are the same as in Fig. 1.

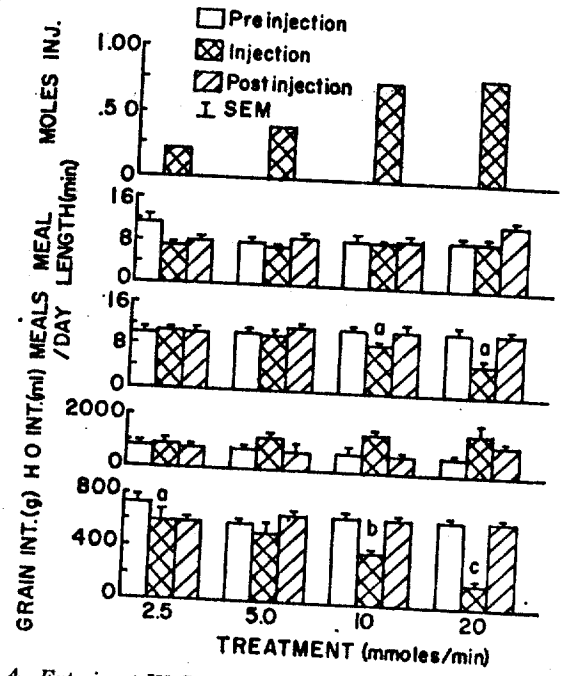

FIG. 4. *Experiment IV.* Solutions (pH = 6.5) of a mixture of sodium acetate (55%), propionate (30%), and butyrate (15%) were injected intraruminally into goats during spontaneous meals. Data are expressed as daily means of 2-day periods. Denotations of significance are same as in Fig. 1.

mmoles/min during eating. Lactate, on the other hand, was a strong depressant of feed intake relative to the other metabolites tested. The response was quite variable, but in some trials the lactate became toxic. Unlike the recovery from the response from acetate, propionate, or butyrate, the recovery from the higher rate of lactate was especially slow. The injection of neither 6.67 mmoles/min of glucose nor of 6.67 mmoles/min of glucose plus 5 mmoles $NaHCO_3$ alone caused a significant depression of feed intake (Fig. 6).

Rumen fluid data. The pH of the rumen fluid increased with increasing rates of injection of all the solutions tested except glucose (Table 1). During the injection of the volatile fatty acids the total concentration of these acids of the rumen fluid tended to increase at the slower rates of injection and to decrease with the higher levels relative to the control periods. The molar proportion of acetate, propionate, or butyrate, when injected alone during an experiment, tended to increase in proportion to the other acids. This was especially true for butyrate for which the proportion nearly tripled during the highest rate of butyrate injection. Although the pH of the rumen fluid increased markedly ($P < .01$) with increasing rates of injection of the mixture of acetate, propionate, and butyrate, neither the average total concentration of volatile fatty acids nor the molar proportions changed appreciably with different treatments. Neither total volatile fatty acid concentration nor molar proportion was significantly affected by the injections of glucose, although the total volatile fatty acid concentration and propionate proportion tended to increase and the acetate proportion to decrease.

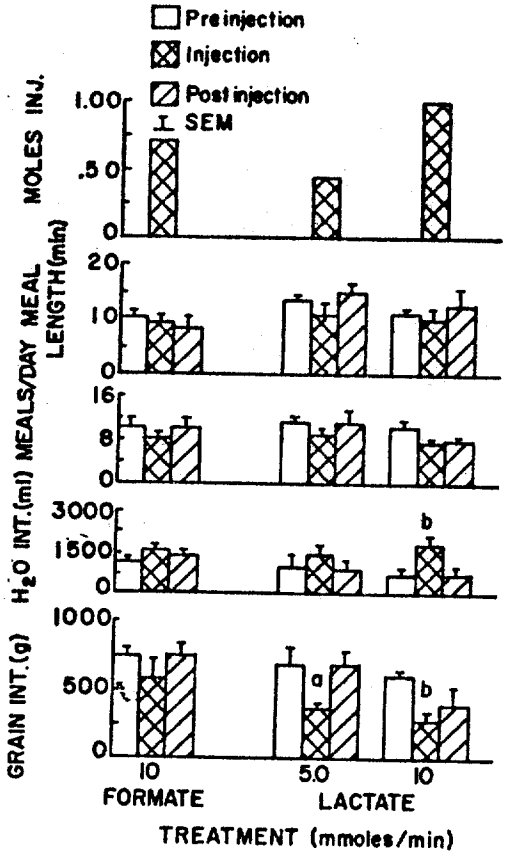

FIG. 5. *Experiment V.* Solutions of sodium formate or lactate (pH ≐ 6.5) were injected intraruminally during spontaneous meals. Denotations of significance are same as in Fig. 1.

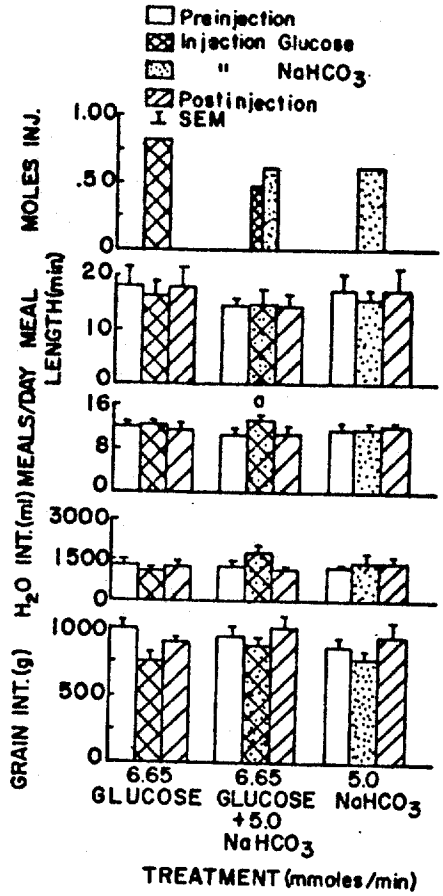

FIG. 6. *Experiment VI.* Solutions of 1.33 M glucose, 1.33 M glucose plus 1 M $NaHCO_3$ or 1 M $NaHCO_3$ were injected intraruminally in goats during spontaneous meals. All data are presented as daily means of 2-day periods. Denotations of significance are same as in Fig. 1.

There were no apparent differences between the effects of the injections of propionate or of propionic acid on pH, molar proportion, or concentration of the volatile fatty acids of the rumen fluid. The pH of the rumen fluid tended to increase with increasing rate of propionate injection although insufficient data were collected to estimate pH or concentrations of the volatile fatty acids of the rumen fluid of goats during the 20-mmoles/min treatment. The concentration of volatile fatty acids tended to decrease with increasing rates of propionate injection; changes in the proportion of the different acids of the rumen fluid were not marked.

DISCUSSION

The data from these experiments clearly show that a close relationship exists between the rate of injection of acetate, propionate, or a mixture of volatile fatty acids and the depression of feeding. The rate of production of acetate, propionate, and butyrate in the rumen under fed conditions has been reported to be about 4.23, 1.17, and 0.97 mmoles/min, respectively, for sheep (22). Therefore, the injection rates of the present experiments are probably near the physiological rates of production following introduction of readily fermentable feedstuffs, except perhaps the 20-mmoles/min treatments. It is quite likely that the feeding-depressant effect of the volatile fatty acids may play a significant role in the overall regulation of energy balance since *1)* they are important energy sources to the ruminant, *2)* their rate of production increases with feeding, and *3)* they are the first and most immediate products of the digestive process to be absorbed. Also produced in the rumen in substantial amounts, at least under certain conditions, are lactate (27, 28) and formate (21). These metabolites are not usually very important energy sources for ruminants, but lactate especially is produced at high rates for a short period of time after readily digestible carbohydrates are ingested (27). On these bases the tests with formate and lactate were done. Glucose is readily fermented in the rumen and was of interest in the present experiments as a test of specificity of the other intermediary metabolites.

These experiments differ from most previous tests of the effect of intermediary metabolites on feeding in ruminants in that the injections were given only during eating of spontaneous meals. In experiments with goats (5, 7, 9) and sheep (10) receiving injections of acetate during spontaneous meals, depressions of feed intake were comparable when similar doses were tested. Experiments with propionic acid given intraruminally over a feeding period lasting several hours caused a feeding depression in sheep (18, 34), whereas tests with cattle have failed to show this effect (24, 28) although sodium propionate injections caused a feeding depression in cattle (29). It has been suggested that the injection of a solution containing both acetate and propionate resulted in no

TABLE 1. *Data from rumen fluid samples*

Experiment	Treatment	N	pH	VFA, mM	Acetate, %	Propionate, %	Butyrate, %
[illegible]te	0	36	5.55	83	52	35	13
	2.5	10	5.85	85	62‡	28†	10†
	5	9	5.97†	98†	56	31	13
	10	10	6.22‡	73	67‡	24‡	9‡
	20	8	6.51‡	60‡	64‡	25‡	12
Propionate	0	12	5.93	82	55	32	13
	2.5	3	5.93	89	57	28	15
	5	11	6.16*	67†	55	33	12
	10	4	6.64‡	71	52	39	10
	20						
Butyrate	0	27	5.56	80	52	35	13
	2.5	7	5.84‡	82	53	31	15
	5	9	6.17‡	70*	54	29†	17†
	10	8	6.01‡	74	48†	27‡	23‡
	20	10	6.44‡	67‡	45‡	21‡	35‡
VFA	0	15	6.03	69	58	31	11
	2.5	7	5.98	90†	59	32	10
	5	8	6.42‡	65	62	25	12
	10	7	6.66‡	79	62	28	11
	20	8	6.90‡	61	67†	24*	9
Formate	0	8	5.62	71	59	32	9
	10	11	6.51†	68	57	31	12*
Lactate	0	12	5.72	72	58	28	14
	5	7	6.58‡	55*	57	28	15
	10	7	6.74‡	54*	55	34*	10†
Glucose	0	15	5.65	77	51	35	11
	6.67	11	5.53	91*	47	40	11
Glu[illegible] + N[illegible]CO_3	6.67	9	5.75	91*	49	42	9
$NaHCO_3$	5.0	13	6.26‡	75	59	29	12

Treatments are designated by millimoles per min injected. * $P < .10$, level of significance between the 0 and injection treatments (*t* test). † $P < .05$. ‡ $P < .01$.

depression of feed intake in dairy cattle (13), which is in contrast to the effects observed with a mixture of volatile fatty acids in studies on sheep (18), or dairy cattle (29, 34), or in the present studies with goats. Those injections resulting in no depression were given in one dose 1 hr prior to a feeding period, and the cattle had been conditioned to a single 6-hr feeding period per day.

Previous tests with butyrate (24, 29, 31, 32) and lactate (30, 24) injected intraruminally during a daily feeding period showed a small depressant effect in cattle and sheep fed mainly roughages. Our experiments differ both in that the injections were given during spontaneous meals and a grain ration was fed. Formate injections into the stomachs of sheep in a single dose did not depress feeding (25, 26).

Volatile fatty acid concentration of the rumen fluid generally decreased with increasing degree of feeding depression. From the data of Table 1 it is not possible to determine the change in volatile fatty acid concentration of the rumen fluid during a meal, but it can be concluded that the depressant effect was not due to a general elevation of a single or of tot[illegible]olatile fatty acids between meals, since the samples were [illegible]n when the feed was removed. The only sustained elevation, apparently, was during meals, and because of decreased feed intake, the average concentration of volatile fatty acids in the rumen fluid decreased between meals. Butyrate was not as effective a depressant even though there was a sustained increase of butyrate concentration of the rumen fluid. The increase in concentration may have been the result of the more nearly normal feed intake, and/or the slower uptake or metabolism of butyrate than of acetate or propionate. The proportion of acetate increased during *experiment I* although the concentration of acetate was little changed. During *experiment IV*, although the proportion of acids was little affected, the acetate proportion tended to increase. The pH of the rumen fluid increased as increasing amounts of Na^+ were injected. In no case was there evidence that the goats developed sustained unphysiological levels of rumen fluid volatile fatty acids or rumen fluid pH.

Although the injection of Na^+ resulted generally in increased oral water intake, the feed-intake depression of the sodium salts of organic acids was apparently not related to the increased Na^+ level. Solutions of propionic acid or sodium propionate had similar feeding-depressing effects. During propionic acid treatments the oral water intake was decreased. Other evidence is the lesser depressant effects of solutions of sodium butyrate and $NaHCO_3$ per millimole injected as compared with acetate and propionate. Previous experiments have also shown the depressant effect of the volatile fatty acids not to be simply an osmotic effect (10, 34). We have recently shown that the increased oral water intake would not inhibit feed intake of goats fed a concentrated diet (9).

The regression lines and correlation coefficients of the percent depression of feed intake and the millimoles injected per meal were calculated. Percent depression of the treatment period as compared with the intake of the control was selected in that the feed intakes of each animal serve as its own control. The millimoles injected per meal were calculated by dividing the moles injected for the 2-day treatment period by the number of meals. The latter parameter is useful in that it is an expression of the effectiveness of the metabolite as a function of meal pattern, and the individual goat's meal pattern is taken into account. The regression equations and correlation coefficients of the treatments were: for acetate—$Y = .39X - 5.18$, N = 24, $r = .76$, $P < .01$; for propionate—$Y = .36X - .27$, N = 18, $r = .69$, $P < .01$; for butyrate—$Y = .17X + 11.40$, N = 24, $r = .50$, $P < .02$; for VFA (mixture of acetate, propionate, butyrate)—$Y = .38X + 8.74$, N = 16, $r = .79$, $P < .01$; for lactate—$Y = 29 + .30X$, N = 8, $r = .30$, $P > .1$; for formate—$Y = 20 + .03X$, N = 6, $r = .02$, $P > .9$; for glucose—$Y = 15 + .30X$, N = 6, $r = .27$, $P > .6$; for glucose plus $NaHCO_3$—$Y = .35X - [illegible]3$, N = 6, $r = .52$, $P > .3$. It can be seen that the slopes of the treatments of acetate, propionate, or volatile fatty acid mixture are all similar while butyrate is clearly less.

In Fig. 7 are shown the regression lines for the millimoles injected per meal and percent depression of feed intake corrected for the estimated digestible energy of the injection solutions (the heat of combustion of the free acids of the solutions injected was converted to estimated equivalents of feed; the digestible energy of the feed was estimated to be 3 kcal/g). The slopes of these lines were less than those of the respective lines of the uncorrected feed-intake

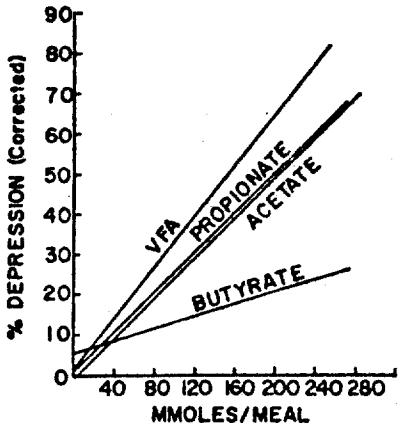

FIG. 7. Regression lines are shown of percent depression of 2-day injection periods corrected for estimated digestible energy of the salt injected as compared with 2-day preinjection periods versus millimoles salt injected per meal. For acetate: $Y = .26X - 1.67$, $N = 24$, $r = .51$, $P < .02$; for propionate: $Y = .25X - 2.37$, $N = 18$, $r = .67$, $P < .02$; for butyrate: $Y = .08X + 5.1$, $N = 24$, $r = .27$, $P > .10$; for VFA (mixture of acetate, propionate, butyrate): $Y = .312X + 3.15$, $N = 16$, $r = .74$, $P < .01$.

data. The correlation coefficient of the butyrate data was not significantly different from 0. Figure 7 clearly shows the relationship of ruminal intermediary metabolites and feeding and that the depression of feed intake is not merely a compensation for the energy of the solutions injected. These regression equations are, of course, applicable only to the conditions under which these tests were done, although it is quite probable that butyrate would not be as strong a depressant as acetate, propionate, or a mixture of volatile fatty acids injected intraruminally independent of the type of ration fed. The small butyrate depression observed in *experiment III*, after correcting for the digestible energy injected, was comparable to that observed with $NaHCO_3$.

Acetate and propionate have nearly the same effect whereas, again, butyrate is quite ineffective. We postulated that there were receptors on the lumen side of the rumen that were sensitive to acetate concentration changes of the rumen fluid since intravenous injections of acetate are much less of a depressant than intraruminal injections (5). We have further shown that an increase in the concentration of acetate of the rumen fluid, but with only a small change in volume, depresses feed intake more than just an increase in the quantity of acetate and an increase in overall volume of the rumen (9). In contrast, we have found that propionate injection in the jugular vein during spontaneous meals is a more effective feeding depressant than intraruminal injections (2). It is also true that injections in the ruminal vein cause a greater depression than similar injections in the jugular vein (2). This is evidence that goats may sense the body state of propionate differently than that of acetate; there may be receptors (possibly in the liver) sensitive to propionate in addition to possible receptors in the rumen wall. These experiments clearly show that acetate and propionate reduce feeding when only small amounts are added to the rumen. Butyrate injected intraruminally is quite ineffective as a depressant, and therefore apparently the sensing mechanisms for acetate and propionate are not very sensitive to butyrate.

Although lactate was a strong depressant of feeding, glucose injections with or without $NaHCO_3$ did not cause a significant depression, especially when the estimated digestible energy of the glucose was taken into account. Glucose would be expected to cause first an increased production of lactate and second of the volatile fatty acids (20, 27, 33). The purpose of the glucose plus $NaHCO_3$ solution was to buffer the possible decreased pH following fermentation of glucose. Under the conditions of these experiments, introduction of lactate did not cause a sustained increase in the proportion of butyrate as observed in a previous experiment (24).

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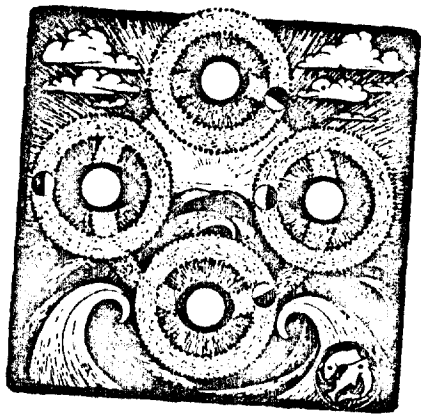

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acceptance* since they were introduced about 20 years ago. They are suitable for yeast-raised as well as other baked goods, and are reasonable in price. Undoubtedly practical trials in the baking industry have provided the mass of technical information for the successful use of these compounds.

Baked Goods Since most molds are destroyed by heat of baking, the contamination of baked goods occurs afterward and growth can flourish under the wrapper in the humid environment. Higher environmental or storage temperatures assist this process. The serious dimensions of the mold contamination problem in bakeries have been outlined by Ingram *et al.*, and others.[126]

One of the earliest reports of the effectiveness of propionates against molds in baked goods was the patent of Hoffman, Dalby and Schweitzer.[121] Ingram (*loc. cit.*) has summarized some of his own data on propionic acid and its sodium and calcium salts in mold control. He found that bread which contained 0.2% of propionate (basis total dough weight) showed no visible mold for over eight days in both the wrapped and unwrapped condition at room temperature. Breads without the preservative molded in four days at the lower temperature and in less than four days at the higher.

A recent study by Seiler presented quantitative data on equilibrium relative humidity as a controlling factor in mold growth on bread and cake, as well as information on propionates and sorbates as mold inhibitors.[211] He reported that, for bread, 0.2% of calcium propionate by flour weight extended mold-free life to six days, as compared with four days for untreated loaves. No odor problems were found at this level.

In relation to rope control by propionate there is the early study of O'Leary and Kralovec.[171] Their data showed 0.11% (basis of flour) of calcium propionate to be more effective than 0.25% of calcium acid phosphate in the control of rope in bread inoculated with various levels of *B. mesentericus*, up to 9,700,000 bacteria per loaf. The propionates appeared to delay the development of color and viscousness more than the phosphate. These workers reported that calcium propionate at a level of 0.188% effectively inhibited rope in bread having a pH as high as 5.8. At pH 5.6, a concentration of 0.15% was effectively inhibitory. Because propionate also inhibits molds and spares yeast, it has superseded the acid phosphate as an anti-rope agent.

More recent comparative data on propionates and other agents for inhibition of rope in baked goods appears in the paper by Ingram and his associates.[126]

Both sodium and calcium propionate can be used in baked goods. The calcium salt is preferred in bread as the calcium contributes to enrichment. The sodium salt is preferred for cakes and unleavened goods where the calcium ion can interfere with chemical leavening. The propionates disperse easily in basic dough ingredients. They do not alter color, taste, volume or baking time at the levels commonly used.

Table 7 shows quantities of propionate recommended for different types of baked goods. These levels may have to be varied with the season, as microbial growth is accelerated in warm weather.

Dairy Products The earliest investigations of the mold-controlling action of propionates for a dairy product appear to be those of Miller and Ingle on the dip process for Cheddar cheese.[129,125] Miller reported that immersion of Cheddar cheese cuts in 8% propionic acid solutions increased the mold-free life at 60°F from the usual 3 to 5 days to 12 to 28 days varying with

* Sales increased from 12.8 million pounds in 1956 to 31 million pounds in 1961.[11]

TABLE 7
Suggested Levels of Propionates in Baked Goods*

Type of Baked Goods	*Level of Propionate*
White breads, buns, rolls, specialties	2½ to 5 oz per 100 lb flour. Three oz usually should be sufficient, but 5 oz may be needed under extreme conditions, such as warm moist weather.
Dark breads, whole or cracked wheat, rye breads, buns, rolls, etc.	3 to 6 oz per 100 lb flour. Four oz usually should be sufficient, but 6 oz may be needed under extreme conditions.
Angel Food Cake	1½ to 3½ oz per 100 lb batter; generally 2½ oz suffices.
Cheese Cake	2 to 4 oz per 100 lb batter; generally 3 oz suffices.
Chocolate or Devil's Food Cake	5 to 7 oz per 100 lb batter; generally 6 oz suffices.
Fruit Cake	2 to 6 oz per 100 lb batter; the higher the fruit content, the less propionate needed.
Pound Cake, White or Yellow Cake	4 to 6 oz per 100 lb batter; generally 5 oz suffices.
Pie Crust	2 to 5 oz per 100 lb dough.
Pie Fillings	2 to 5 oz per 100 lb filling.

In cakes use only sodium propionate. Add it with the baking powder and take out an equivalent weight of salt from the formula.
In fillings add propionate just before the end of cooking.
In pie crust dissolve propionate in the water to make up the crust.
"Brown 'n serve" rolls. Many bakers today prefer an oil spray on these rolls in order to allow the rolls to expand without tearing and, at the same time, to reduce the formation of a tough skin. Sodium propionate may be incorporated into the spray mixture in the following proportions.

Vegetable oil	15.0 lbs
Lard or vegetable shortening	5.0 lbs
Mono- and di-glyceride emulsifiers	0.8 lbs
33% solution of sodium propionate	1.0 lbs

(quantities of vegetable oil and lard or vegetable shortening may be varied according to the spray and flow characteristics desired)

*Source: Chas. Pfizer & Co., Inc., 1966, Brochure, "Food Preservatives."

the cheese sampled, contamination, length of immersion and length of drainage. Somewhat more concentrated sodium or calcium salts were required, as might be expected.

For butter, propionate-treated parchment wrappers were found to give considerable protection in experiments by Macy and Olson.[144,172] However, the use of this additive for butter has not been officially accepted.

Currently, sodium or calcium propionate, or combinations of these, are permitted under Federal Standards of Identity in cheese and cheese products shown in Table 8.

Propionates are added to the starting materials in the process cheese cooker. With pasteurized process cheese and cheese products, the propionates may

be added before, or along with, the emulsifying salts. When used with cold pack, propionates may be mixed with other ingredients and added in this manner or may be sprinkled into the ground cheese base while it is being agitated and worked.

Miscellaneous Uses From 0.2% to 0.4% sodium propionate has been said to inhibit the growth of molds on the surface of malt extract. Wolford and Anderson have reported use of propionates to retard development of mold on syrup, blanched apple slices, figs, cherries, blackberries, peas and lima beans.[259]

TABLE 8
Types of Cheese and Cheese Products Which May Contain Propionates

Section of Code of Federal Regs., Part 21	*Type of Cheese Product*
19.750	Pasteurized process cheese
19.751	Pasteurized blended cheese
19.755	Pasteurized process cheese with fruits, vegetables or meats
19.760	Pasteurized process pimento cheese
19.763	Pasteurized blended cheese with fruits, vegetables or meats
19.765	Pasteurized process cheese food
19.770	Pasteurized process cheese food with fruits, vegetables or meats
19.787	Cold-pack cheese food
19.788	Cold pack cheese food with fruits, vegetables or meats

Food additive regulations in Finland mention permissible use of propionates in non-sterilized, hermetically closed canned foods. Regulations for Sweden include permissible use in carbonated beverages.

Handling and Storage

Sodium and calcium propionates are neutral salts and present little hazard in storage. They will emit acrid fumes if heated to high temperatures.[207]

Assay

Propionates may be determined in breads and other baked goods by procedures given in *Official Methods of Analysis of the A.O.A.C.*, 1965 ed., p. 204.

Sulfur Dioxide and Sulfites

Sulfur dioxide has been used in foods for many centuries. According to the review by Joslyn and Braverman, fumes of burning sulfur were used as a sanitizing agent in wine-making by the ancient Egyptians and Romans.[133] The practice is continued by the modern wine industry. Sulfiting* has been applied to other foods, both abroad and in the United States, an important use being in dehydrating of fruits and vegetables.[209] During the 19th century

* Sulfiting is used in this chapter to mean the use of either sulfur dioxide or sulfites.

ignite spontaneously so that these substances should be kept away from cellulosic materials. Sorbic acid and potassium sorbate can cause eye irritation. In case of contact with the eyes, flush with water for 15 minutes and get medical attention.

Assay

Assay methods for sorbic acid and its salts in foods are not included in the 1965 edition of *Official Methods of Analysis of the A.O.A.C.* Various methods have been published in the literature.

Alderton and Lewis described a spectrophotometric method which is a modification of one developed earlier by Melnick and Luckmann.[3,152] Ciaccio has applied spectrophotometry to the determination of sorbate in low-calorie salad dressing which also contains propyl-*p*-hydroxybenzoate, and Harrington *et al.*, have used this principle in the determination of sorbic acid in apple cider.[47,108] By combining chromatographic methods with spectrophotometry, Höyem was able to separate and quantitatively determine sorbic acid, benzoic acid and the methyl, ethyl, propyl and butyl esters of *p*-hydroxybenzoic acid.[123]

Spanyár and Sánder have reported a titrimetric method for sorbates in foods, and Nury ond Bolin have described a colorimetric procedure.[223,168]

Other assay methods are mentioned on pp. 150–151 under *p*-hydroxybenzoic acid esters.

Propionic Acid and Its Salts

Propionic acid is a member of the aliphatic monocarboxylic acid series. The antimicrobial action of the members of this series has been known for some time, one of the earliest reports being that of Kiesel in 1913.[134] Another broad-scale study was that by Hoffman, Schweitzer and Dalby.[122] They evaluated the fungistatic properties of the normal saturated fatty acids containing from 1 to 14 carbon atoms, over a pH range from 2 to 8 and found that antifungal effectiveness varied according to chain length, concentration of acid and pH. These same investigators in that year patented the use of propionic acid and certain of its salts as a mold inhibitor in bread.[121] Propionates were selected because the higher homologs, although they have higher antimicrobial activity, also have tastes and odors which would be noticeable, especially in baked goods.

At about this same time others found that propionic acid and propionates are especially effective against *Bacillus mesentericus*, which causes "rope" in bread,[171] and also that propionates are useful to retard the molding of cheese.[159]

Physical and Chemical Properties

Propionic acid, a liquid, has a strong odor and is somewhat corrosive. Therefore, the propionates used in the food industry as antimicrobials are the sodium and calcium salts. These yield the free acid in the pH range of the foods in which they are used. These salts have a slight, cheese-like flavor, which blends with that of most foods.

Both of these salts are white, free-flowing, readily soluble powders. The sodium salt is more soluble, dissolving at the rate of 150 gm per 100 ml of water at 100°C and in alcohol at the rate of 4 gm per 100 ml at 25°C.

Calcium propionate dissolves in water at the rate of 55.8 gm per 100 ml at 100°C and is insoluble in alcohol. Both the sodium and calcium salt

blend readily with emulsifying agents used in processed and blended cheese, as well as with basic dough ingredients and in filling for pies.

CH_3CH_2COONa Sodium Propionate $(CH_3CH_2COO)_2Ca$ Calcium Propionate

Specifications for sodium and calcium propionate appear in *Food Chemicals Codex.*

Antimicrobial Activity

Propionates are more active against molds than sodium benzoate, but have essentially no activity against yeast. They have little action also against bacteria with the notable exception of their ability to inhibit the organism which causes rope. The pH for optimum activity of propionates ranges up to 5.0, although in some foods they are active up to pH 6, or slightly higher. The effect of variations in pH of medium on antimicrobial activity has been studied by Cruess and Richert,[54] Cruess and Irish[53] and Olson and Macy.[172] The relative activity of sodium propionate against 7 species of bacteria, 3 yeasts and 2 molds in broth culture has been reported by Wolford and Anderson.[250]

Safety for Use

Propionic acid occurs naturally in Swiss cheese at levels which may be as high as 1%.[11] It is also a metabolite within the rumen of ruminant animals.[66] Propionate is metabolized like other fatty acids in the mammalian body, as shown by studies in which the acid was labeled with radioactive carbon.[260]

In rat feeding trials, Harshbarger found that sodium or calcium propionate had no effect on growth when fed at levels of from 1 to 3 gm. daily, for 4 to 5 weeks, beginning when the animals were about 4 weeks of age.[111] In later studies by Graham *et al.*, a level of 5% of propionate was placed in bread before baking and the finished product was fed as three-fourths of the diet of rats for one year.[96] There was no discernible toxicity in spite of the simultaneous presence of three other common bread additives at high concentrations in the diet.

Regulatory Status

Under Federal Regulations propionates are generally recognized as safe for use in foods and no upper limits are imposed, except for bread, rolls and cheese which come under Standards of Identity. Sodium and calcium propionate are limited to 0.32% of flour in white bread and rolls and 0.38% in the corresponding whole wheat products (CFR 17.1, 17.5). In cheese products they are limited to 0.3%.

Propionates are not mentioned in the food additive regulations of France, West Germany, Greece, Portugal and Turkey, but they are included in those of other West European countries for which information is at hand, as well as those of Canada.

Applications

The major uses of propionates are to prevent mold and rope in baked goods and mold in certain types of cheese. Published experimental data in technical journals are meager, but the propionates have received widespread

CHRONIC TOXICITY OF BREAD ADDITIVES TO RATS. PART II

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In an earlier investigation[1] of the chronic toxicity of certain bread additives the ingredients tested (chlorine dioxide, polyoxyethylene (8) monostearate, sodium propionate and a mixture of propyl gallate and butylated hydroxyanisole) were incorporated in bread at 50 times the normal concentration and used for 52 weeks at 75 per cent. of the diet of rats. Under the conditions of the experiment no adverse effects were noted and these results were at variance with some of the experimental evidence obtained by other investigators, particularly in regard to the polyoxyethylene (8) monostearate[2,3,4,5]. Possibly this difference was due to experimental technique. It was of interest, therefore, to retest the bread ingredients previously studied, with the difference that they were not mixed with water, baked into bread, and the bread dried and ground but were added directly to the basal ration.

Diet I contained the ingredients of an essentially normal bread (75 per cent.) added to a basal medium supplying minerals, vitamins, protein, fat, and bulk (25 per cent.). In diet II, flour treated with 50 times the normal amount of chlorine dioxide, lard containing 50 times the usual level of antioxidants, and sodium propionate and polyoxyethylene (8) monostearate each at 50 times the usual concentration were incorporated in the bread ingredient portion of the diet. Diets III, IV, V and VI were similar to diet II, except that one ingredient in each, respectively emulsifier, mould inhibitor, antioxidants, or flour improver, was reduced to the normal level. Diet VII was a commercial cubed ration ground to a consistency similar to that of the other diets. Diets were mixed at intervals of no greater than 2 weeks and were kept in closed cans in the refrigerator. The details of diet composition for convenience are reproduced from the previous publication[1] in Table I.

A group of 210 Wistar strain male albino rats ranging in age from 23 to 36 days and in weight from 35 to 79 g. was divided into 14 sub-groups by arranging them in descending order of weight and randomly assigning the rats in successive lots of 14 to the 14 sub-groups. These sub-groups, each consisting of 15 animals, were assigned to the 7 diets yielding two series, called for convenience replicates A and B, and numbered according to the number of the diet given. The oldest rats had been maintained on diet VII from weaning at 22 days of age until placed on test. The 14 groups of rats were housed in cages with wire screen floors. Each cage held 1 group (15 rats). Cages were moved in a regular rotation on the racks so that all spent approximately equal intervals at the 4 different levels above the floor. Food and water were permitted *ad libitum*. Records of weekly weight, food consumption and mortality

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TABLE I

Diet compositions

Part A.—75 per cent. of the diet. Ingredient	Diet I	II	III	IV	V	VI	VII
Commercial bread flour[1]	70·5	—	—	—	—	70·5	GROUND MASTER
Bread flour 50 × ClO_2[1]	—	70·5	70·5	70·5	70·5	—	
Commercial lard[2]	1·8	—	—	—	1·8	—	
Lard 50 × Antioxidants[2]	—	1·8	1·8	1·8	—	1·8	
Sodium propionate[3]	0·1	5·0	5·0	0·1	5·0	5·0	
Polyoxyethylene (8) monostearate[4]	0·3	15·0	0·3	15·0	15·0	15·0	
Cerelose (glucose)	20·4	0·8	15·5	5·7	0·8	0·8	
Salt (NaCl)	1·8	as I	as I	as I	as I	as I	
Skim milk powder	2·2	"	"	"	"	"	
Malt flour	0·5	"	"	"	"	"	
Yeast food[5]	0·2	"	"	"	"	"	
Wytase[6]	0·9	"	"	"	"	"	
Yeast	1·3	"	"	"	"	"	
	100·0	100·0	100·0	100·0	100·0	100·0	

B.—25 per cent. of the diet. Ingredient	Diet I	II	III	IV	V	VI	
Casein	60·0	as I	as I	as I	as I	as I	FOX CUBES
Alphacel[7]	9·4	"	"	"	"	"	
Corn oil	12·8	"	"	"	"	"	
Mineral mix[a]	12·0	"	"	"	"	"	
Vitamins and excipient[b]	5·8	"	"	"	"	"	
	100·0	100·0	100·0	100·0	100·0	100·0	

[a] Minerals—the 12·0 g. of mineral mix present in 100 g. of basal diet were made up as follows:—

KCl	1·580	$FePO_4$	0·195
KH_2PO_4	4·100	$KAl(SO_4)_2.12H_2O$	0·001
$Ca_3(PO_4)_2$	1·970	$CuSO_4.5H_2O$	0·080
$MgSO_4$	1·190	NaI	0·076
$MnSO_4.H_2O$	0·027	KI	0·001

[b] Vitamins—the 5·8 g. of vitamins and excipient in 100 g. of basal diet were made up as follows:

Thiamine hydrochloride	0·0020 g.	Folic acid	0·0008 g.
Riboflavine	0·0040 g.	*p*-Aminobenzoic acid	0·0040 g.
Calcium pantothenate	0·0080 g.	Liver fraction‡	0·8000 g.
Pyridoxine	0·0020 g.	Menadione	0·0004 g.
Nicotinamide	0·0080 g.	E concentrate*	0·0560 g.
i-Inositol	0·0800 g.	A and D concentrate†	0·1600 g.
Choline chloride	0·4000 g.	Corn oil	4·2830 g.

* Vitamin E concentrate, 350 mg. of *d*, α-tocopheryl acetate equivalent per g.
† Navitol, 65000 A, 13000 D per g.
‡ Wilson's Liver fraction L.

NOTES

[1] Chlorine dioxide.—Canadian No. 1 patent flour was treated with 0·3 g. of chlorine dioxide per barrel (196 lb.) to yield commercial bread flour and with 15·0 g. of chlorine dioxide per barrel to yield high ClO_2 flour.

[2] Antioxidants.—Tenox II, a commercial preparation containing 20 per cent. of butylated hydroxyanisole, 6 per cent. of *n*-propyl gallate, 4 per cent. of citric acid and 70 per cent. of propylene glycol was used at the rates of 0·05 per cent. in commercial lard and 2·5 per cent. in high antioxidant lard.

[3] Propionate.—The commercial propionate sold by Du Pont de Nemours and Co., under the trade name Mycoban was used.

[4] Polyoxyethylene monostearate.—The material sold by Atlas Powder Co. as polyoxyethylene (8) monostearate under the trade name Myrj 45 was used.

[5] Yeast food.—The commercial material sold under the name RKD by Standard Brands was used.

[6] Wytase.—This material is sold by J. R. Short Canadian Mills, Ltd., and is used commercially to improve whiteness.

[7] Alphacel is a commercial cellulose supplying bulk without nutritive value.

were kept. Where the condition of the carcass warranted it, detailed autopsies were performed on animals that died on test; in other instances advanced autolytic changes precluded accurate decision as to the cause of death.

After 16 weeks on the diets 2 rats selected from each group by random choice were killed and a number of tissues were preserved for histological

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study. Blood samples from each of these rats were analysed for hæmoglobin content by a slight modification of the pyridine hæmochromogen method of Rimington[6]. After 32 weeks on the diet only 3 rats survived in group II A and the experiment was terminated. All surviving rats were killed and the liver, left kidney, heart and spleen were weighed. Tissue samples were reserved for histological examination and subsequently sections from cerebellum, cerebrum, myocardium, trachea, œsophagus, thyroid, salivary gland, thymus, peri-bronchiolar lymph gland, lung, stomach, small intestine, liver, spleen, adrenal, pancreas, kidney, bladder and testis were studied.

Results

The mean body weights at intervals during the 32-week feeding trial of the rats in the 14 groups are presented graphically in Figure 1. The mean body weights of the survivors on each diet and the standard errors of the means are shown for replicates A and B and for the replicates combined in Table II.

TABLE II

Final weight of survivors at 32 weeks
Weight in g. ± standard error

Diet	Replicates combined		Replicate A		Replicate B	
	Mean weight	Number	Mean weight	Number	Mean weight	Number
I	296 ± 7·4	16	297 ± 12·4	7	295 ± 9·6	9
II	258 ± 7·4	13	257 ± 21·8	3	259 ± 13·2	10
III	268 ± 10·3	15	277 ± 13·5	7	260 ± 15·4	8
IV	281 ± 9·4	21	273 ± 14·2	11	290 ± 10·9	10
V	252 ± 7·0	21	247 ± 10·0	11	258 ± 9·0	10
VI	263 ± 6·5	18	255 ± 10·0	10	274 ± 7·0	8
VII	292 ± 8·1	19	305 ± 16·2	7	283 ± 8·4	12

TABLE III

Analysis of variance of final weight data

Main effect	DF	Mean square	F
Replicates	1	335	1
Diet	5	4,345	4·4*
Replicates X diet	5	792	1
Error	111	984	—

* Significant at P = 0·01.

Growth was depressed, particularly during the first few weeks on diets II, III, V and VI, i.e. on those diets containing the high level of sodium propionate. A detailed examination of the data revealed that the total weight gain subsequent to the first 4 weeks on the diet was approximately the same for each diet and the apparent depression of the final weights on these 4 diets high in propionate was the result of the curtailed growth during the first few weeks. Growth rates based on weight changes between the 5th and 32nd weeks were not significantly influenced by diet. Growth on diet I was very similar to that on diet VII throughout the test.

Statistical analysis of the final weight data for diets I to VI using Snedecor's methods for disproportionate groups[7] revealed a significant

(P = 0·01) influence of diet on final weight (Table III). The effect was mainly that of the high level of sodium propionate, for growth on diet IV was not significantly depressed.

Cumulative feed consumption per rat per day over the 32-week period is shown in Figure 2. As in the previous study[1] the rats ate more of the stock diet (VII) than of any of the test diets. In this experiment, feed consumption figures for all diets were higher than the corresponding

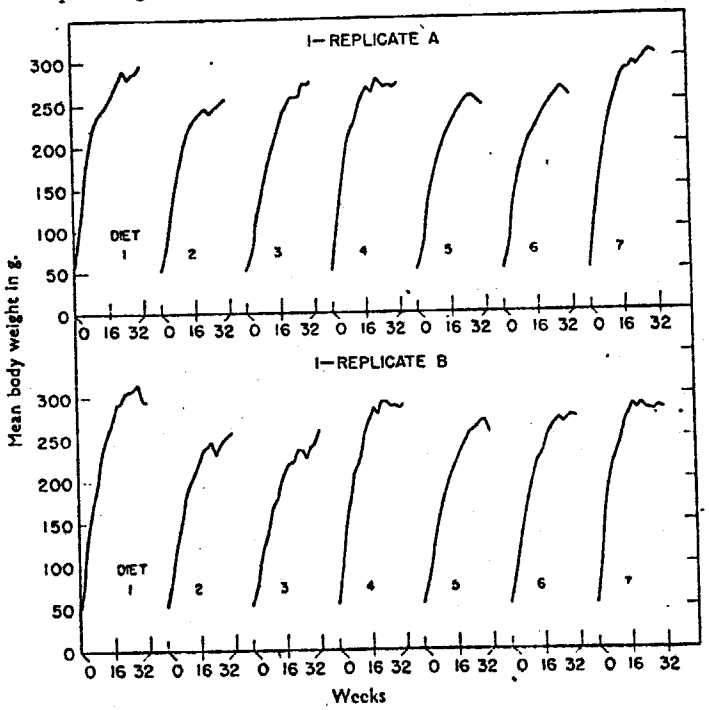

Fig. 1. Mean body weight of the rats in the 14 groups.

figures from the earlier data by an average of some 8·5 per cent. Mean body weights overall were only 3 per cent. higher. These differences are reflected in the generally poorer feed efficiency also depicted graphically in Figure 2. The striking effect of diet which may be observed here is the very poor feed efficiency during the first weeks on diets II, III, V and VI, particularly on diet III.

Curtailed growth, slightly lower feed consumption, and markedly lowered feed efficiency on diets II, III, V and VI during the early weeks indicate that sodium propionate fed at high levels to the young rat had a deleterious effect. That this effect was not solely due to lack of palatability of the diet is obvious from the fact that feed efficiency was much

more seriously affected than was feed consumption. Although the data are not amenable to statistical assessment, they suggest that polyoxyethylene (8) monostearate at high levels in conjunction with high sodium

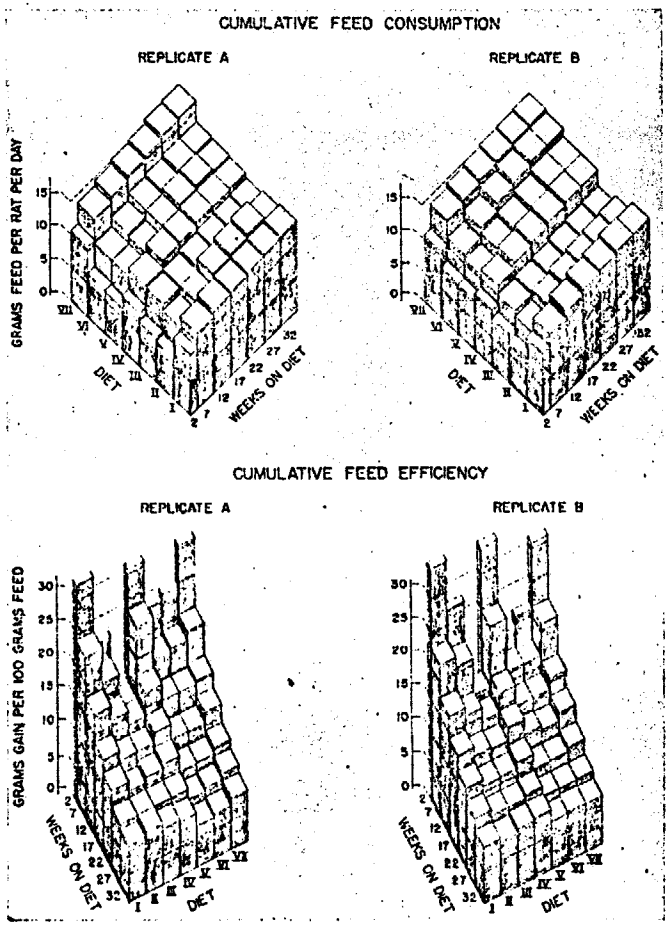

FIG. 2. Cumulative feed consumption per rat per day over the 32-week period.

propionate (diets II, V and VI) increased the efficiency with which the high propionate diet was utilised by young rats (compare diet III). Apparently the rats became acclimatised to the high propionate diet, for growth rate, feed consumption and feed efficiency approached the control levels within a few weeks.

The hæmoglobin levels in the blood of the 28 rats killed at the end of 16 weeks were all within the normal range for rats in this colony.

Sections from the tissues of 151 rats were stained with hæmotoxylin and eosin and examined microscopically. As in the previous investigation[1] the groups to which the individual rats belonged were unknown

TABLE IV

HISTOPATHOLOGICAL FINDINGS

	Replicate													
	A							B						
Diet	I	II	III	IV	V	VI	VII	I	II	III	IV	V	VI	VII
Number of rats examined*	9	5	9	13	13	12	9	11	12	10	12	12	10	14
Normal	4	2	4	2	0	1	1	5	4	1	5	1	1	3
Trachea—inflammation	–	–	–	–	5	2	–	2	–	1	–	4	1	–
Lung—respiratory infection	2	1	–	5	10	7	6	2	1	1	3	8	7	6
Lung—papilloma of bronchus	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Thymus—inflammation	–	–	1	–	–	1	–	–	–	–	–	–	–	–
Stomach—gastritis	–	–	1	–	–	–	–	–	2	–	–	–	–	–
Stomach—erosion	–	–	–	–	–	–	–	–	1	–	–	1	–	–
Adrenal—congestion	–	–	–	1	–	–	–	–	–	1	–	–	–	–
Kidney—tubal necrosis	–	–	–	–	1	–	–	–	–	–	–	–	–	–
Kidney—nephritis	–	1	–	1	1	–	–	–	–	–	–	1	–	–
Kidney—congestion	–	–	–	–	–	–	–	–	–	1	–	1	–	–
Liver—cloudy swelling	–	–	1	2	1	–	–	–	–	–	2	–	–	–
Liver—venous congestion	–	–	–	1	–	–	–	–	1	–	–	–	–	–
Pancreas—fatty degeneration of acinar tissue	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Intestine—enteritis	–	–	1	–	1	–	–	–	1	–	–	–	–	–
Intestine—papilloma	–	–	–	–	–	–	–	–	–	1	–	–	–	–
Testis—reduced spermatogenesis	–	–	–	–	–	–	–	1	–	–	–	–	–	–
Bladder—parasitic cysts	4	3	5	8	5	6	8	5	5	9	6	4	6	8

* 2 rats were killed at 16 weeks, the remainder at 32 weeks.

MORTALITY DATA

Died on test	6	10	6	2	2	3	6	4	3	5	3	3	5	1
Autopsy	3	4	5	2	0	2	3	2	1	4	2	3	4	0
Undiagnosed	3	6	1	0	2	1	3	2	2	1	1	0	1	1
Respiratory infection	1	4	2	–	–	2	1	1	1	4	2	3	3	–
Middle ear disease	2	–	3	1	–	–	–	1	–	–	–	–	2	–
Encephalitis } Probable extension from middle ear infection	2	–	4	–	–	–	–	–	–	–	–	–	–	–
Meningitis } Probable extension from middle ear infection	–	–	1	1	–	–	–	1	–	–	–	–	2	–
Pericarditis	–	–	1	–	–	–	1	–	–	–	–	–	1	–
No visible lesion	–	–	–	1	–	–	1	–	–	–	–	–	–	–

to the pathologist at the time the tissues were studied. The histopathological findings are presented in Table IV and have been summarised by the pathologist as follows:

Findings in the cerebrum, cerebellum, myocardium, œsophagus, thyroid, salivary gland, thymus, small intestine, spleen, adrenal, pancreas, stomach and testis were negative or of no significance. Most of the lungs exhibited some changes due to pneumonia, including emphysema, bronchiectasis and atelectasis, although only acute and sub-acute cases have been listed in Table IV. The predominant bronchopneumonia invariably was accompanied by mild chronic inflammatory changes in the trachea. The two instances in which congestion and polymorphonuclear infiltration of the peribronchiolar lymph nodes were observed probably were the result of infection from the lungs. While cloudy swelling of the liver is the first sign of a degenerative process caused by

bacterial or chemical toxins, the few cases noted here were very mild and of doubtful significance. The incidence of kidney abnormality is similar to that reported previously[1], and again these abnormalities were observed in rats on diets II, III, IV and V, those diets high in chlorine dioxide. The incidence of the kidney changes again is too low to warrant any conclusion. The lung changes, which are of bacterial origin, and the bladder parasites (*Trichosomoides crassicauda*) occur normally in this rat colony. From a histopathological standpoint the tissues were essentially negative as far as consistent changes due to a dietary toxicant are concerned.

TABLE V

EFFECT OF DIET ON THE WEIGHT OF VARIOUS ORGANS
Mean tissue weight in mg./100 g. of rat

Diet	Number	Liver	Left kidney	Heart	Spleen
Replicate A—					
I	7	35·4	3·94	3·79	3·26
II	3	38·4	4·07	3·73	3·20
III	7	33·2	3·87	3·46	3·19
IV	11	36·0	3·76	3·54	2·71
V	11	35·7	4·05	3·65	2·85
VI	10	31·7	4·13	3·59	2·84
VII	7	36·3	3·56	3·39	2·69
Overall standard deviation		4·1	0·39	0·33	0·55
Replicate B—					
I	9	27·3	3·66	3·39	3·04
II	10	32·9	3·86	3·62	2·71
III	8	34·3	4·21	3·53	2·94
IV	10	30·0	3·48	3·49	2·68
V	10	32·4	3·83	3·65	2·62
VI	8	32·2	3·91	3·60	2·45
VII	12	30·9	3·56	3·73	2·74
Overall standard deviation		4·5	0·40	0·37	0·45

The mortality data also are presented in Table IV. Those deaths for which no explanation is given occurred at night and autolysis was too far advanced to permit a valid diagnosis. As in the previous study[1], it is confidently believed that most of these deaths may be attributed to respiratory infection. The high mortality in group II A appears to have been due to a particularly severe outbreak of respiratory infection in this cage. It is possible, of course, that the high level of all 4 potential toxicants in diet II predisposed these rats to infection, but the lack of abnormally high mortality in the corresponding group II B argues against such a possibility. In general, it may be concluded that the variations in mortality were of no significance in so far as they might reflect toxicity of the diet.

The mean weights of the livers, left kidneys, hearts and spleens of the rats surviving until killed after 32 weeks on the diets are presented in Table V. For statistical analysis the data summarised in Table V were transformed into percentage of the mean weight for each tissue and analysed by Snedecor's methods for analysis of variance with disproportionate subclass numbers[7]. The results of these analyses are presented in Table VI. Since the diet X replicate interaction was significant in one instance, and was of appreciable magnitude in the other 3 cases, these interactions were used in the calculation of F for diet and replicate. On

this basis spleen weights were significantly different (P = 0·05) between replicates. No explanation of this finding can be offered: it is suggested that this may be one of the 5 out of 100 cases where the difference is due to chance.

TABLE VI

ANALYSIS OF VARIANCE ON TISSUE WEIGHT DATA

Main effect	D.F.	Liver		Left kidney		Heart		Spleen	
		M.S.	F.	M.S.	F.	M.S.	F.	M.S.	F.
Replicates ..	1	2,005·	3·6	252	1·1	250	1·1	3,000	6·7*
Diet	5	314	< 1·0	470	2·1	102	< 1·0	742	1·7
Diet X replicates	5	560	4·1*	221	2·1	229	2·5	447	1·8
Error	111	137	—	107	—	90	—	249	—

* Significant at P = 0·05.

DISCUSSION

The transient growth-depressing effect of a high level of sodium propionate in the diet of young rats in this experiment is contrasted with the lack of influence of this compound in the previous study[1]. As was noted at the time, baking the bread and later drying it resulted in a strong odour of propionate and it is probable that the final concentration of propionate in the diets of the previous study was considerably reduced. Since the present diets were unheated, little or no propionate was lost and the material at this higher level in the diet exerted an early growth-depressing effect. Obviously since normal rates of growth were resumed after a few weeks and no pathology attributable to propionate poisoning was detected, this effect of propionate cannot be regarded as too serious. In the human dietary, propionate is used almost entirely in baked goods and it is likely that the baking process would materially reduce any potential hazard.

In agreement with the previous findings, the high levels of the antioxidants butylated hydroxyanisole and propyl gallate, of the emulsifier polyoxyethylene (8) monostearate and of the flour improver chlorine dioxide had no detectable deleterious effect on the growth of rats over a 32-week feeding period. In spite of the change in experimental method, 11·25 per cent. of polyoxyethylene (8) monostearate in the diet failed to exert deleterious effects.

SUMMARY

1. Bread ingredients containing 50 times the normal concentration of chlorine dioxide, propyl gallate and butylated hydroxyanisole, or polyoxyethylene (8) monostearate given as 75 per cent. of the diet for 32 weeks did not harmfully affect growth or mortality of male rats. Sodium propionate at the same high level caused a depression of growth during the first few weeks of feeding, but did not influence mortality.

2. The high concentration of polyoxyethylene (8) monostearate, sodium propionate, antioxidants and chlorine dioxide had no detectable effect on hæmoglobin levels in the blood, on organ weights, or on histopathology of the tissues.

3. No evidence was obtained that the simultaneous presence of a high concentration of more than one potential toxicant in the diet led to any synergistic action.

The authors wish to thank Mr. Robert Van Burek and Wallace and Tiernan, Ltd., for the treatment of the flour with chlorine dioxide, the Tennessee Eastman Corporation for supplies of the antioxidant Tenox II, the J. R. Short Canadian Mills, Ltd., for a supply of Wytase, and the Lake of the Woods Milling Co. for a quantity of malt flour. They are indebted to Miss E. Connell and Mr. H. Teed for technical assistance, and to Dr. D. G. Chapman for the hæmoglobin determinations.

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A METHOD FOR THE DETERMINATION OF 2-CARBETHOXYTHIO-1-METHYLGLYOXALINE

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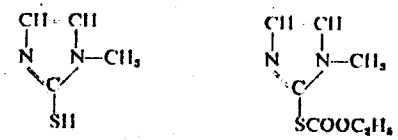
Previous papers[1,2] described the estimation of the antithyroid substance 2-mercapto-1-methylglyoxaline (I) by means of the chloroimide reaction, as well as the detection of various 2-mercaptoglyoxalines with the iodobismuthous acid test. In the former procedure, small amounts of the compound in a buffer solution (*p*H 8·0) are treated with 2:6-dichloroquinone-chloroimide, which results in the formation of yellow condensation products. At this *p*H the reaction product is extractable with chloroform, a property which makes the reaction specific for 2-mercaptoglyoxalines as well as thiouracils[3].

We have been examining recently the possibility of applying these methods to the estimation of 2-carbethoxythio-1-methylglyoxaline (II) in tissues and body fluids, and the present communication is part of this work. The 2-carbethoxythio-derivative is an antithyroid substance with a prolonged action which was introduced by Lawson, Rimington and Searle[4].



(I) (II)

Procedure

Reagents: (1) Solution of 2:6-dichloroquinone-chloroimide 0·4 per cent. in aldehyde-free absolute ethanol. The solution will keep for about 4 weeks if stored in a dark bottle.

(2) N Hydrochloric acid.

(3) N Sodium hydroxide.

(4) Standard solution of 2-carbethoxythio-1-methylglyoxaline. Dissolve 100 mg. of the pure compound in about 190 ml. of distilled water and make to 200 ml. The solution keeps indefinitely.

(5) Standard solution of 2-mercapto-1-methylglyoxaline. Dissolve 100 mg. of the pure compound in 100 ml. of water. The solution keeps indefinitely.

(6) Buffer-chloride solution, *p*H 8·0. To 50 ml. of 0·2 M boric acid in 0·2 M potassium chloride, add 4 ml. of 0·2 N sodium hydroxide, and 100 ml. of a 20 per cent. aqueous solution of sodium chloride. Make to 200 ml. with distilled water. Adjust *p*H to 8·0 with 0·1 N sodium hydroxide.

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Studies of Pharmacological and Toxic Actions of Propionates

Examination of General Pharmacological Actions and Toxicity of Sodium Propionate and Calcium Propionate

Saburo Hara, Takeshi Shibuya, Keido Yakazu, Tatsunori Kobayashi, Ryoji Takahashi, Takako Takeuchi and Ken Tokizaki (Department of Pharmacology. Tokyo Medical College under the Directorship of Professor Saburo Hara).

(For a brief English synopsis, refer to the original text on p.261)

I. Introduction

Along with the improvement and progress of food in recent years, there has been a mounting concern for food additives, food preservatives, condiments and nutrients, etc. These substances are added to food in order to preserve its freshness, prevent the aging or degeneration thereof or bring about a favorable effect in giving a shape thereto.

Among these, propionates are used in the preparation of bread and cookies. Their addition improves the finishing of the bread and cookies; they raise the effect of the finished food product to prevent mildew formation, prevent the growth of Rope bacterium and aavorably effect the preservation of foods. When they are added to the animal feed , they are known to give a favorable effect to the preservation of such food.

Propionates are prepared in the following manner: BY using carbohydrates (dextrose), organic acids (lactic acid), polyvalent alcohols, etc. as the substrates fermentation is carried out under anaerobic conditions by propioni bacterium. The fermentation solution is either ether-extracted or organic acids are collected by water-vapor distillation. A salt is added to the propionic acid which is obtained by fractionation. These include sodium propionate which is prepared by adding sodium carbonate to a propionate and calcium propionate which is prepared by adding calcium carbonate to the same and others.

These substances are whit crystalline powders or granular powders, having a slight odor. The solubility of sodium propionate is 10.8 milligrams per 100 milliliters of water and that of calcium propionate is 3.6 milligrams per 100 milliliters of water.

The above-described information has been clarified in the following publications: A general book published by Katagiri and Ichikawa on the fermentation of propionic acid, the result of a test on the bacterium-resistant character of preservatives published by the Fermentation Research Laboratory of the Industrial Technology Institute (2), a report published by the Food Research Bureau of the Agriculture and Forestry Ministry on the effect of propionates upon bread making and mildew prevention (3), the general book writes by Nakae (4) on the relationship between mildews and Rope and the problems of sodium propionate as an agent for the prevention of mildews as reported in Shiryo Jiho (Report on Feeds), some general articles appearing in the journals concerningfood, the report on the test for the inhibition of Rope bacterium and mildew as conducted by the Research Laboratory of Sankyo Company, Limited the report of L. R. Richarson on the causes and prevention of the temperature elevation of feeds and the researches and studies conducted by the Research Department of Nippon Yeast Company, Limited. The present authors concur with these results in view of the other reports and publications written on the subject.

As food additives and food preservatives, it is anticipated that propionates will be used every day and, therefore, there is need to strictly examine their toxicity, if any, from the standpoint of public health.

In our department, we have been carrying out a long-term experiment on the feeding of various chemicals and food additives on a continuous basis to various animals and rats, in particular, inorder to know their effects upon the general conditions, including growth and body weight (6-15). Particularly

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in the case where the use of the substance in question appears to be for a long period of time, it is necessary to examine their toxicity to a sufficient extent.

Nevertheless, there are no reports on the long-term examination of the toxicity of these propionates, including sodium propionate and calcium propionate, in particular, nor are there reports on their physiological and pharmacological effects.

Under these circumstances, the present authors examined the presence or absence of the toxicity of these substances and their general pharmacological effects on the basis of the growth curves which are most closely related to the development of toxicity and carried out the experiments for the purpose of offering the results as a reference material to be used in the general application (of the same) to food.

II. Experimental Materials and Experimental Method

The materials used in the experiment were the propionates offered by Nippon Yeast Company, Limited; these included sodium propionate (which may hereafter be abbreviated as S.P.) and calcium propionate (which may herefter be abbreviated as C.P.). Their chemical structures are shown in Table 1.

It is mentioned in this connection that the animals used in the experiments included toads, mice, rats and rabbits. The experimental method will be explained in detail in each section dealing with the experimental result.

(Table 1 Chemical Structures of C. P. and S. P.)

III Experimental Result

(A) Examination of the Presence or Absence of Toxicity

1. Experiment on the Acute Toxicity of C.P. and S.P.

By using male mice of the DD system, whose body weight was in the neighborhood of 15 grams, LD_{50} was obtained in accordance with the Behrens-Kaerber process. The result obtained is shown in Table 2. (Reference should be made to Table 2.)

(Table 2 Table showing the toxicity of C.P. and S.P. on mice. a. Method of administration b. Substance to be tested c. Administration into the vein d. Administration into the abdomenal cavity e. Hypodermic administration f. Administration through the mouth.)

In the case of an injection into the vein, C.P. was in the amount of 580 milligrams per kilogram and S.P. was in the amount of 1,380 milligrams per kilogram. As for the injections into the abdomenal cavity, it was 2,250 milligrams per kilogram in S.P. and 720 milligrams per kilogram in C.P.. As regards the hypodermic administration, it was 1,020 milligrams per kilogram in C.P. and 3,200 milligrams per kilogram in S.P. In the case of an administration through the mouth, it was 3,340 milligrams per kilogram in C.P. and 5,100 milligrams per kilogram in S.P.

In the case of administrations through the mouth, in particular, themovement of mice 5' afterward was reduced by the administration of 6,000 milligrams per kilogram, with a staggering pace developing gradually. In the neighborhood of 15', the animals assumed a crawling attitude on their stomachs and, after 20', there were observed some cases where rapid respirations appeared in conformity with the forceful backward positioning. In the neighborhood of 1^h 20', the eye balls popped out and white turbidity was observed, and this was followed by death.

In the case of C.P., further, a course which was similar to the administration of S.P. was observed when C.P. was administered in an amount of more than 2,800 milligrams per kilogram, with death coming at the end. In both cases, however, a large amount was required.

Between C.P. and S.P. however, it seems that the toxicity of C.P. is stronger.

2. Experiment on the Chronic Toxicity of C.P. and S.P.

By using male rats of the Wister system, a chronic toxicity test was carried out for a period of six months (180 days).

In the case where a substance to be tested is administered to rats for the purpose of examining its toxicity, it is necessary that the examination of such a substance whose toxicity is assumed to be small be carried out on the basis of a growth curve and that a detailed observation be carried out on the general conditions. In order to obtain the most suitable growth curve, it is desirable that the experimental animals be uniformalized as to their system and that they be healthy in a certain stage of growing. Therefore, the present authors raised and observed approximately twice the necessary number of male rats belonging to the Wister system for a period of more than a week in a constant-temperature laboratory and only those whose general conditions were satisfactory were selected for use in the experiments.

As for the method of feeding and raising, a constant-temperature raising case of the Koyama formula was used in a constant-temperature room, the temperature for their raising was constantly amaintained at 22 degC plus/minus one degC, with one rat being accommodated in one cage and 10 rats beingconsidered as a group. As for the feed used, Oriental Powder Feed N. M. F. was used and it was administered in accordance with the standard of 15 grams per 100 grams of body weight. In addition, an aqueous solution of combined vitamins was added to drinking water at the rate of one gtt/100 milliliter per day. In addition, fresh vegetables were given once in a period of seven days.

(Table 3-1 Table Showing Changes in the Weight of Untreated Control Rats A. Group I a. Date of Experiment b. Number of Experiment Days c. Temperature for Raising (degC) d. Body weight of rats e. Average f. Amount of Vegetables Fed g. Date of Experiment h. Number of Experiment Days i. Temperature for Raising j. Body weight of rats (g) k. Average m. Amount of Vegetables Fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Date Administration Started.)

(Table 3-1 Table Shows Changes in the Weight of Untreated Control Rats A. Group I a. Date of Experiment b. Number of experiment days c. Temperature for Raising, degC d. Body temperature of rats e. Average f. Amount of Vegetables Fed g. Date of Experiment h. Number of Experiment days i. Temperature for Raising j. Body weight of Rats(g) k. Average m.Amount of Vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(Table 3-2 Table Shows Changes in the Weight of Untreated Control Rats A. Group I a. Date of Experiment b. Number of Experiment Days c.Temperature for Raising degC d. Body temperature of rats e. Average f. Amount of Vegetables fed g. Date of Experiment h. Number of Experiment Days i. Temperature for Raising. j. Body weight of Rats (g) k. Average m. Amount of Vegetables Fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10.)

(Table 3-2 Table shows Changes in the Weight of Untreated Control Rats. A. Group I a. Date of Experiment b. Number of Experiment Days c. Temperature for Raising, degC d. Body temperature of rats e. Average f. Amount of vegetables fed g. Date of experiment h. Number of Experiment Days i. Temperature for Raising j. Body weight of rats (g) k. Average m. Amount of Vegetables Fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(Table 4-1 Table shows Changes in the Weight of Rats given feed containing 1% Calcium Propionate for 180 days continuously by month. A. Group II a. Date of Experiment b. Number of Experiment Days c. Temperature for Raising, degC d. Body temperature of rats e. Average

f. Amount of Vegetables Fed g. Date of Experiment h. Number of Experiment Days i. Temperature for Raising j. Body weight of rats (g) k. Average m. Amount of vegetables Fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Date Administration started.)

(Table 4-1 Table Showing Changes in the Weight of Rats given Feed containing 1% calcium Propionate for 180 days continuously through the mouth A. Group II a. Date of Experiment b. Number of Experiment Days c. Temperature for Raising(degC.) d. Body weight of rats e. Average f. Amount of Vegetables Fed g. Date of Experiment h. Number of Experiment Days i. Temperature for Raising j. Body weight of Rats (g) k. Average m. Amount of Vegetables Fed n. Cabbage 10 o. Cabbage10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(Table 4-2 Table shows Changes in the Weight of Rats given feed containing 1% calcium Propionate continuously for 180 days through the mouth. A. Group II a. Date of Experiment b.Number of Experiment Days c. Temperature for Raising, degC d. Body temperature of rats e. Average f. Amount of Vegetables Fed g. Date of Experiment h. Number of Experiment days i. Temperature for Raising j. Body weight of Rats (g) k. Average m. Amount of Vegetables Fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10.)

(Table 4-2 Table Shows Changed in the Weight of Rats given feed containing 1% calcium Propionate continuously through the mouth A. Group II a. Date of Experiment b. Number of Experiment days c. Temperature for Raising, degC d. Body temperature of rats e. Average f. Amount of vegetables fed g. Date of Experiment h. Number of Experiment Days i. Temperature for Raising j. Body weight of Rats (g) k. Average m. Amount of Vegetables Fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q.Cabbage 10 r. Cabbage 10 s. Cabbage 10.)

(Table 5-1 Table shows changes in the weight of rats given food containing 3% calcium propionate continuously for 180 days through the mouth. A. Group II a. Date of Experiment b. Number of Experiment Days c. Temperature for Raising, degC d. Body temperature of rats e. Average f. Amount of vegetables fed g. Date of experiment h. Number of experiment days i. Temperature for Raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Date administration started.)

(Table 5-1 Table shows changes in the weight of rats given food containing 3% sodium propionate continuously for 180 days in the mouth A. Group III a. Date of exerpiment b. Number of Experiment days c. Temperature for Raising, degC d. Body temperature of rats e. Average f. Amount of Vegetables Fed g. Date of experiment h. Number of Experiment days i. Temperature for Raising j. Body weight of rats (g) k. Average m. Amount of Vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(Table 5-2 Table showing changes in the weight of rats given food containing 3% calcium propionate continuously for 180 days through the mouth A. Group III a. Date of experiment b. Number of experiment days c.Temperature for Raising (degC) d. Body weight of rats e.Average f. Amount of vegetables fed g. Date of experiment h. Number of experiment days i. Temperature for Raising j. Body weight of rats (g) k. Average m. Amount of Vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10.)

(Table 5-2 Table shows changes in the weight of rats given food containing 3% calcium propionate continously for 180 days through the mouth A. Group III a. Date of experiment b. Number of Experiment days

c. Temperature for Raising, degC d. Body temperature of rats e. Average f. Amount of Vegetables fed g. Date of experiment h. Number of experiment days i. Temperature for Raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(Table 6-1 Table shows changes in the weight of rats given food containing 6% calcium propionate continuously for 180 days through the mouth. A. Group IV a. Date of experiment b. Number of Experiment days c. Temperature for Raising, degC d. Body temperature of rats. e. Average f. Amount of vegetables fed g. Date of experiment h. Number of Experiment days i. Temperature for Raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Date administration started.)

(Table 6-2 Table shows changes in the weight of rats given food containing 6% calcium propionate continuously for 180 days through the mouth A. Group IV a. Date of experiment b. Number of Experiment days c. Temperature for Raising, degC d. Body temperature of rats e. Average f. Amount of vegetables fed g. Date of experiment h. Number of Experiment Days i. Temperature for Raising j. Body weight of rats (g) k. Average m. Amount of Vegetables Fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(Table 6-2 Table shows changes in the weight of rats given food containing 6% calcium propionate continuously for 180 days through the mouth A. Group IV a. Date of experiment b. Number of experimental days c. Temperature for raising, degC d. Body temperature of rats e. Average f. Amount of Vegetables fed g. Date of experiment h. Number of experiment days i. Temperature for raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10

r. Cabbage 10 s. Cabbage 10.)

(Table 6-2 Table showing changes in the weight of rats given food containing 3% calcium propionate continuously for 180 days through the mouth A. Group [illegible] a. Date of experiment b. Number of experiment days c. Temperature for raising (DegC) d. Body weight of rats e. Average f. Amount of vegetables fed g. Date of experiment h. Number of Experiment days i. Temperature for raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(Table 7-1 Table shows changes in the weight of rats given food containing 1% sodium propionate continuously for 180 days through the mouth A. Group [illegible] a. Date of experiment b. Number of experiment days c. Temperature for raising, degC d. Body temperature of rats e. Average f. Amount of vegetables fed g. Date of experiment h. number of experiment days i. Temperature for raising j. Body weight of rats (g) k. Average m. Amount of Vegetables fed n. Cabbage 10 o. Cabbage 10 p. cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Date administration started.)

(Table 7-1 Table shows changes in the weight of rats given food containing 1% sodium propionate continuously for 180 days through the mouth A. Group [illegible] a. Date of experiment b. Number of experiment days c. Temperature for Raising, degC d. Body temperature of rats e. Average f. Amount of vegetables fed g. Date of experiment h. Number of experiment days i. Temperature for raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(TABLE [illegible] Table shows changes in the weight of rats given food containing [illegible] sodium propionate continuously for 180 days through the mouth A. Group [illegible] a. Date of Experiment b. Number of Experiment days

c. Temperature for raising, degC d. Body temperature of rats e. Average f. Amount of vegetables fed g. Date of experiment h. Number of experiment days i.Temperature for raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10.)

(Table 7-2 Table shows changes in the weight of rats given food containing 1% sodium propionate continuously for 180 days through the mouth A. Group V (2) a. Date of experiment b. Number of experiment days c. Temperature for raising, degC d. Body temperature of rats e. Average f. Amount of vegetables fed g. Date of experiment h. number of experiment days i. Temperature for raising j. body weight of rats (g) k. Average m. Amount of vegetables fed n. cabbage 10 o. cabbage 10 p. cabbage 10 q. cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(Table 8-1 Table showing changes in the weight of rats given food containing 3% sodium propionate continuously for 180 days through the mouth A. Group VI a. Date of experiment b. Number of experiment days c. Temperature for raising (degC) d Body weight of rats e. Average f. Amount of Vegetables fed g. Date of experiment h. Number of experiment days i. Temperature for raising j.Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Date administration started.)

(Table 8-1 Table shows changes in the weight of rats given food containing 3% sodium propionate continuously for 180 days through the mouth A. Group VI a. Date of experiment b. Number of experiment days c. Temperature for raising, degC d. Body temperature of rats e. Average f. amount of vegetables fed g. Date of experiment h. Number of experiment days i. Temperature for raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. cabbage 10 p. cabbage 10

q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(Table 8-2 Table shows changes in the weight of rats given food containing 3% sodium pripionate continuously for 180 days through the mouth. A. Group VI a. Date of experiment b. Number of experiment days c. Temperature for raising degC g. Date of experiment h. number of experiment days i. Temperature for raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o.cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10.)

(Table 8-2 Table shows changes in the weight of rats given food containing 3% sodium propionate continuously for 180 days through the mouth A. Group VI a. Date of experiment b. Number of experiment days c. Temperature for raising, degC d. Body temperature of rats. e. Average f. Amount of vegetables fed g. Date of experiment h. Number of experiment days i. Temperature for raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(Table 9-1 Table shows changes in the weight of rats given food containing 6% sodium propionate continuously for 180 days through the mouth A. Group VII a. Date of experiment b. Number of experiment days c. Temperature for raising, degC d. Body temperature of rats e. Average f. Amount of vegetables fed g. Date of experiment h. Number of experiment days i. Temperature for raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10. r. Cabbage 10 s. Cabbage 10 t. Day administration started.)

(Table 9-1 Table showing changes in the weight of rats given food containing 6% sodium propionate continuously for 180 days through the mouth A. Group VII a. Date of experiment b. Number of experiment days c. Temperature for raising (degC) d. Body weight of rats e. Average f. Amount of vegetables fed g. Date of experiment

h. Number of experiment days i. Temperature for raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

(table 9-2 Table shows changes in the weight of rats given food containing 6% sodium propionate continuously for 180 days through the mouth A. Group VII a. Date of experiment b. Number of experiment days c. Temperature for raising, degC d. Body temperature of rats e. Average f. Amount of vegetables fed g. Date of experiment h. number of experiment days i. Temperature for raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10.)

(Table 9-2 Table shows changes in the weight of rats given food containing 6% sodium propionate continuously for 180 dats through the mouth A. Group VII a. Date of experiment b. number of experiment days c. Temperature for raising, degC d. Body temperature of rats e. Average f. Amount of vegetables fed g. Date of Experiment h. Number of experiment days i. Temperature for raising j. Body weight of rats (g) k. Average m. Amount of vegetables fed n. Cabbage 10 o. Cabbage 10 p. Cabbage 10 q. Cabbage 10 r. Cabbage 10 s. Cabbage 10 t. Cabbage 10.)

Feed containing one, three and six per cent of the substance to be tested was used. As for the standard for its administration, the calculation was made beforehand on the following basis:

According to the research and study of Nippon Yeast Company, limited, from 0.09 to 0.18 grams of propionates is added to 100 grams of bread products. Therefore, the toxicity examination was carried out on the maximum amount of 0.2 grams in our department. If it is assumed that the amount of bread consumed by the individual per day is 150 grams, the amount of propionates which an individual whose body weight is 60 kilograms consumed every day

will be 0.3 grams per 60 kilograms (which is five milligrams per kilogram). This amount was used by the present authors as the standard amount.

As regards the quantitative relationship of administrations among the various groups which are given food containing one, three and six per cent, these are 300 times, 900 times and 1,800 times the standard amount per rat on an average. In other words, the experiment was carried out by dividing the animals into seven groups, depending upon the amount of the administration per day, as follows:

(1) First group: Untreated and control group

(2) Second group: administered feed containing one per cent of C.P.

(3) Third group administered feed containing three per cent of C.P.

(4) Fourth group: administered feed containing six per cent of C.P.

(5) Fifth group: administered feed containing one per cent of S.P.

(6) Sixth group: administered feed containing three per cent of S.P.

(7) Seventh group: administered feed containing six per cent of S.P.

In this manner, the body weights of the rats were measured every day by using an automatic scale having a sensitivity of 0.5 grams (the measurement being carried out at the specified time each day). At the same time, the state of the intake of both feed and water was observed.

In addition, the state of the movement of rats, their appetite, the state of their hair conditions, the presence or absence of skin diseases, the presence or absence of nose bleeding, changes in the eyes and other general conditions were observed.

After feeding on a continuous basis for a period of 120 days, a blood collection was carried out immediately for the purpose of a blood test, the rats were caused to die through hemorrhage, the weights and volumes of various organs were measured and those values were contrasted with those of the control.

[illegible] formally for the purpose of

carrying out a pharmacological and histological examination.

(**Table 10** The number of blood corpuscles, the amount of blood pigment, the pigment index and the percentage of white corpuscles on the 131 st day of rats belonging to various groups in the case where calcium propionate and sodium propionate were fed for a period of 180 days on a continuous basis. 1. Group of rats 2. Number 3. Sex 4. Body weight 5. Number of corpuscles 6. Number of red blood corpuscles 7. White blood corpuscles. 8. Blood pigment (per cent) 9. Pigment index 10. Percentage of white blood corpuscles 11. Untreated control group 12. Average 13. Average 14. Average 15. Average 16. Average.)

(continuation of Table 10 1. Classification of rats 2. Number 3. Sex. 4. Body weight, gram 5. Blood corpuscles 6. Number of red blood corpuscles 7. Number of white blood corpuscles 8. Blood pigment 9. Blood pigment index 10. Percentage of white blood corpuscles 11. Average 12. Average.)

1. Experiment on Growth in the case where feed containing various percentages of C.P. and S.P. was given for a period of 180 days on a continuous basis.

(1) First group: Control group fed with untreated feed

The rats belonging to the control group which were fed feed containing no such substance but under the same feeding conditions as those belonging to the other groups where feed included substances to be tested showed an average increase in weight from 90.6 grams to 325.9 grams 180 days later or upon the completion of the experiment. During this period of time, the rats showed no abnormalcy in their movement, appetite and hair conditions, etc. There was observed no nose bleeding and no change in the eyes and their scale was extremely satisfactory.

By using the body weight curve for the untreated group and the general

conditions obtained as the standard, the various groups which were given samples were compared and examined. (Refer to Table 3 and Figures 1 and 3.)

(2) Second Group: In the case where feed containing one per cent of C.P. was given.

The average body weight of rats, which was 92.1 grams at the start of the feeding of samples, increased to 338.9 grams 180 days afterward. During this period of time, the body weight curve showed a gradual increase every day. In the weight curve, there is observed a passing drop in the weight of all of the animals tested on the 103rd day. This was caused by a leakage of the water supply. There was observed no abnormalcy thereafter. In the general conditions, there is no mentionable abnormalcy in this group as compared with the control group. Rather, the average values after 90 days were better than those of the untreated control group. (Reference should be made to Table 4 and Figures 2 and 3.)

(3) Third Group: In the case where feed containing three per cent of C.P. was given.

The body weights of rats increased every day after the feeding of the sample started. The average body weight of 10 rats, which was 93.0 grams at the start of the feeding, increased to 342.2 grams 180 days thereafter. During this period of time, no abnormalcy whatsoever was observed. Only in two cases, there was observed a marked increase in body weight on the 80th day and 110th day. The curve representing the average body weight was better than that of the untreated control group. In this group, furthermore, the rate which was the best among all the group which were fed C.P. was shown. (Reference should be made to Table 5 and Figures 3 and 3.)

(4) Fourth group: In the case where feed containing six per cent of C.P. was given.

The body weight of rats increased every day in a gradual manner after the

feeding started, and a satisfactory growth was shown. The average weight of 10 rats, which was 92.2 grams at the start of the feeding, increased to 337.8 grams 180 days thereafter. During this period of time, no development of any disease symptoms was found. There was a slight tendency after the passage of 50 days for the difference to grow bigger between those whose weight increase was marked and those others whose weight increase was normal, with the curve starting to show some width between the upper quarter and the lower quarter. However, the average body weight was found to be better than that of the untreated control group and that of the group fed with one per cent C.P. (Reference should be made to Table 6 and Figures 4 and 8.)

(5) Fifth group: In the case where feed containing one per cent of S.P. was given.

The weights of rats showed a gradual increase every day after the start of feeding. The average body weight of 10 rats, which was 89.7 grams at the start of feeding, increased to 348.5 grams 180 days afterward. During this period of time, no disease or abnormalcy was observed. As compared with the untreated control group, the growth of this group was found to be better. Only in one case, however, there was observed a low rate of growth from around the 28th day. However, recovery was made around the 60th day and its growth thereafter was found to be normal. (Reference should be made to Table 7 and Figures 5 and 8.)

(6) Sixth Group: In the case where feed containing three per cent of S.P. was given.

The body weight of rats showed a normal increase even after the start of feeding. The average body weight of 10 rats, which was 92.9 grams at the start of feeding, increased to 359.1 grams 180 days afterward. During this period of time, no abnormalcy whatsoever was observed. The average curve was the best as compared with that of the untreated control group. In particular,

the rise was quite marked from around 87th day (Reference should be made to Table 8 and Figures 5 and 8.)

(7) Seventh Group: In the Case where feed containing six per cent of S.P. was given.

The body weight of rats showed a gradual increase even after the start of feeding. The average weight of 10 rats, which was 93.4 grams at the start of feeding, increased to 328.0 grams 180 days afterward. During this period of time, there was no development of disease symptoms. Its general conditions were found to be no different from those of the untreated control group. When a comparison is made with the group fed with one per cent or three per cent of S.P., the average curve seems to be slightly less satisfactory. However, the general situation was not different at all. (Reference should be made to Table 9 and Figures 7 and 8.)

2) Examination of the Bloods of rats fed with C.P. and S.P.

On the 181st day, an examination of the bloods of all rats belonging to the various groups fed with C.P. and S.P. and those belonging to the untreated control group was carried out. The result obtained is shown in Table 10. (Reference should be made to Table 10.)

3) Examination of Organ Weights and Volumes in Rats Fed with C.P. and S.P.

After the completion of the experiment on the rats which were administered samples for a period of 180 days, they were all killed by bleeding and the weights and volumes of the cerebrum (brain), the thyroid gland, the adrenal and other various internal organs were measured. The result obtained is shown in Table 11. (Reference should be made to Table 11.)

(Table 11 The weights (gram) and volumes (milliliter) of various internal organs on the 181st day of the rats belonging to various groups which were fed calcium propionate and sodium propionate for a period of 180 days on a combined basis. 1. Classification of rats. 2. Number 3. Sex

4. Body weight (grams). 5. Cerebrum 6. Weight 7. Volume 8. Cerebellum 9. Weight 10. Volume 11. Thyroid gland 12. Weight 13. Volume 14. Stomach 15. Weight 16. Volume 17. Heart 18. Weight 19. Volume 20. Liver 21. Weight 22. Volume 23. Spleen 24. Weight 25. Volume 26. Kidney 27. Right 28. Left 29. Weight 30. Volume 31. Weight 32. Volume 33. Adrenal ("para" kidney) 34. Right 35. Left 36. Weight 37. Volume 38. Weight 39. Volume 40. Testicles 41. Right 42. Left 43. Weight 44. Volume 45. Weight 46. Volume 47. Lung 48. Weight 49. Untreated control 50. Average 51. Average 52. Average 53. Average 54. Average 55. Average 56. Average)

No significant difference was observed between the cases where samples were fed and the control.

4) Pathological and Histological Examination of Rats fed with C.P. and S.P.

Regarding these various organs of rats which were fed with these samples, a pathological and histological examination was further carried out. A strict examination of the presence or absence of any disease symptoms such as an inflammation, degeneration or swelling, etc. was carried out. Figure 9 shows a part of the histological image of the organs of rats fed with the samples. (Reference should be made to Figure 9.)

In other words, after the blood test and a measurement of the weight and volume of each internal organ were carried out, all rats were examined by the naked eye as to the presence or absence of any disease symptom in each organ. Then, each organ was fixed by using formalin and hematoxylin-Erosin dying was carried out for the purpose of effecting a pathological and histological examination.

(Figure 1 Weight curve for rats belonging to the untreated control group. a. Weight of rats b. Number of days passed. c. Administration started.)

(Figure 2 Curve showing the body weights of rats which were given, through the mouth, feed containing one per cent of calcium propionate for a period of 180 days on a continuous basis. a. Weight of rats (g) b. Number of days passed. c. Administration (feeding) started.)

(Figure 3 Curve representing the body weight or rats which were given, through the mouth, feed containing three per cent of calcium propionate for a period of 180 days on a continuous basis. a. Body weight of rats(g). b. Number of days passed c. Administration (feeding) started.)

(Figure 4 Curve representing the body weight of rats which were given, through the mouth, feed containing six per cent of calcium propionate for a period of 180 days on a continuous basis. a. Body weight of rat(g). b. Number of days passed. c. Administration (feeding) started.)

Regarding the examination of each individual organ, there was no abnormalcy in the upper skin cells and the lower structure of the mucous membrane in the case of the alimentary canal, with the normal image being shown. As regards the stomach, the lineal structure was normal, with no wear symptoms being observed. There was observed no fat degeneration and the deposition of lime. There was observed no abnormalcy in the upper skin cells or in the hypodermic structure. No contractive or other inflammations were observed.

In the case of large or small intestines, the upper skin cells and linear cells showed no such degenerative changes as swelling, contraction or destruction, etc. nor was there observed an increase in the number of free cells in the layer intrinsic to the mucous membrane. Even in the case of the structure below the mucous membrane, there was observed no such abnormalcy as will cause one to think of such circulatory obstacles as blood congestion, thrombus, bleeding or swelling, etc.

In the case of the liver, there was observed no such degenerative changes as the [illegible] and degeneration of liver cells, etc. There was no

inflammatory image, with the normal structure of the liver being completely maintained.

In the case of the kidney, there were found no contraction, turbidity, swelling and fat deposition on the kidney structure, nor was there observed any destruction. In the case of "thread balls," in particular, there was observed no such disease symptoms as blood congestion, swelling or degenerative changes, etc.

In the case of the upper skin of the fine urination tube, there was observed no denegeration nor was there any abnormalcy in the formation of circular columns.

There were some cases where a slight deposition of hemosiderin was observed in the case of the spleen. Such a deposition, however, is also found in healthy animals and it can be considered to be physilogical. There was no abnormalcy in the cell increase, blood congestion, and others which are more important.

In the case of the heart, there were observed no such degenerative changes as the fat degeneration, lime deposition or substantive turbidity, etc. There was no congestion of the blood, swelling or bleeding. There was not observed any circulatory obstacle stemming from the formation of thrombus. There was observed no such inflammatory pathological changes as the inflammatory change of the internal membranes of the heart or the inflammation of the myocardium, etc.

In the case of the lung, there were some cases where a slight bronchial inflammation was observed. Since such a situation was observed even in the control group, it cannot be considered a pathological abnormalcy stemming from the substance that was tested. Most of the other cases showed no change whatsoever relative to abscess formation in the lung structure. There was observed no artery blood congestion, other cases of blood congestion or bleeding,

etc. nor was there observed any circulatory obstacle.

In addition to the above, there was observed no obstacle to the growth in the brain. There was no inflammation, degeneration or swelling, etc. In the spleen, no such circulatory obstacles as an inflammation or degeneration were observed.

In the thyroid gland, there were cases where only the follicular swelling was observed. When iron coloring was carried out, in particular, the same could not be considered to be a pathological abnormalcy stemming from the substance tested inasmuch as it was observed in the control group, too. No pathological changes were observed in the adrenal ("para" kidney) and in the testicles.

(Figure 5 Curve representing the body weight of rats which were given, through the mouth, feed containing one per cent of sodium propionate for a period of 100 days on a continuous basis. a. Body weight of rats (g). b. Number of days passed c. Administration (feeding) started.)

(Figure 6 Curve representing the body weight of rats which were given, through the mouth, feed containing three per cent of sodium propionate for a period of 100 days on a continuous basis. a. Body weight of rats(g) b. Number of days passed c. Administration (feeding) started.)

(Figure 7 Curve representing the body weight of rats which were given, through the mouth, feed containing six per cent of sodium propionate for a period of 100 days on a continuous basis. a. Bodyweight of rats (g) b. Number of days passed c. Administration(feeding) started.)

(Figure 8 Comparison of Rat weight curves at various [illegible] propionate [illegible] propionate Given (Average) a. Body weight of rat (g) b. Number of days passed c. Administration (feeding) started.)

(Note: [illegible] control group numbers, such as No.2, No.3, etc.

(B) Examination of General Pharmacholgical Effects

1. Effects of C.P. and s.P. upon the Respirations and Blood Pressure of Rabbits.

The effects upon the respirations and the neck artery blood pressure of rabbits anesthetized by urethane were examined.

In the case of a small amount of C.P. and S.P., there was observed almost no change in the respiration and blood pressure. In the case of the administration of a large amount such as 500 milligrams per kilogram, there was observed in C.P. a lowering of the blood pressure and a temporary stoppage of respirations, with an apparent recovery being made thereafter. Several minutes afterward,stroke-like respirations were observed and the respirations stopped.

In the case of S.P., the blood pressure was temporarily lowered to a slight extent. In this case, the respiration was observed to be temporarily excited with the amplitude and number of respirations being increased. It is mentioned in this connection that no respiratory stoppage was observed at an amount equivalent to 500 milligrams per kilogram which is the amount for the respiration stoppage for S.P. and C.P.

In the case of S.P. the lowering of the blood pressure was observed at 1,000 milligrams per kilogram and the respiration stopped at this time. (Reference should be made to Figures 10 and 11.)

2. Effects of C.P. And S.P. upon the Extracted hearts and the Heart and Smooth Muscles.

Both C.P. and S.P. did not show any marked effect upon the heart and the smooth muscle. There was a tendency, however, for the function of the former to be somewhat greater than the latter.

(Figure 9 Structural images of main organs of rats which were administered (fed) propionate (6 per cent C.P.) on the 181st day. A: Stomach B: small intestines C: Liver D: Kidney E. Heart F:SPleen G: Lung H. Testicles.)

(Figure 10 Effect of S.P. upon the respiration and blood pressure of Rabbits and, particularly, the amount of same for the stoppage of respiration (S.P. 1,000 milligrams per kilogram o.v.).)

(Figure 11 Effect of C.P. upon the Respiration and blood pressure of rabbits and, particularly, the amount of same causing the stoppage of respiration (C.P. 500 milligrams per kilogram i.v.).)

IV General Summary and Observations

To sum up the above experimental results, the following observations can be made:

1) In an "acute" experiment on mice, the LD_{50} is 580 milligrams per kilogram for C.P. and 1,380 milligrams per kilogram for S.P. in the case of an administration into the vein. In the case of an administration into the abdomenal cavity, it is 720 milligrams per kilogram for C.P. and 2,250 milligrams per kilogram for S.P. In the case of a hypodermic administration, it is 1,020 milligrams per kilogram for C.P. and 3,200 milligrams per kilogram for S.P. In the case of administration through the mouth, it is 3,340 milligrams per kilogram for C.P. and 5,100 milligrams per kilogram for S.P.

In view of the fact that, in the case of an administration through the mouth, the development of an acute toxicity requires a considerably large amount of C.P. or S.P., it is assumed that the toxicity of the propionates including C.P. and S.P. is extremely low or nearly non-existent.

2) [illegible] on the rats for a period of 100 days and, during this period of time, the curve representing the body weights of the rats belonging to seven groups was compared and contrasted with the curve for the control group. [illegible] to observe any difference between the curve representing the average growth of C.P. and S.P. and the curve repre-

senting the average growth of the control group. The order of the body weight on the 180th day was three per cent S.P. one per cent S.P., three per cent C.P., six per cent C.P., one per cent C.P., six per cent S.P. and the control, in the order of largeness, all curves being located above the curve of the control group.

Regarding the mutual relationship between C.P. and S.P, C.P. is located in between S.P. and it is difficult to observe the difference between the two. Regarding the concentrations, no significant difference was observed. In view of an observation of these curves and the general conditions, it is difficult to observe the effect of the propionates and no development of toxicity was seen.

3) Regarding the examination of the bloods of rats which were given various amounts of C.P. and S.P. , no abnormalcy was found, as compared with the control, in the number of red blood corpuscles, the number of white blood corpuscles, the amount of blood pigment and the blood pigment index. Even the percentage of the white blood corpuscles was found to be within the range of normal physiological variations. Thus, this was completely found normal. It is believed that the propionates have no effect whatsoever upon the distal blood.

4) The weight and volume of various internal organs of the rats which were fed various amounts of C.P. and S.P. were measured. As a result, it was found that there was no marked difference between the groups which were fed samples and the control group. It is believed that the propionates have no effect upon these internal organs.

5) The aforementioned various organs of the rats was found to have a regular histological image by a pathological and hitological examination. No pathological abnormalcy was found in each of these organs such as an inflammation, degeneration, destruction and swelling, etc. Only in an

extremely few cases, there was found a hemosiderin deposition in the spleen structure and in a few cases, there was observed some follicular swelling in the thyroidal gland. In view of the fact that the control group contained cases having similar symptoms, it was difficult to think that they stemmed from the toxicity of C.P. or S.P. Thus, it is believed on the basis of these pathological and histological examinations that there is no toxicity of the propionates at all.

6) In the general pharmacological examination it was found that the propionates do not show a peculiarly marked effect. The amount required in the case of respirations and blood pressure were large and there was observed almost no effect upon the smooth muscles. In other words, the general effect of C.P. and S.P. are weak and their toxicity is difficult to detect.

V. Conclusion of Experiment and Determination

In the case of the propionates, an extremely large amount of calcium propionate and sodium propionate was found in the acute toxicity test to be required for causing death when administered through the mouth. In other words, their toxicity is believed to be either extremely small or nearly non-existent.

In the chronic toxicity test based on the administration through the mouth of a rat, it was found that the body weight curves were no different from that of the control group both in the case of calcium propionate and in the case of sodium propionate. Rather, these curves were found to be somewhat better. By the feeding of these samples, the growth (of the rats) was not prevented, with normal growth being observed and absolutely no toxicity was observed.

In addition, an examination of the blood and a measurement of the weights and volumes of various internal organs showed no significant difference from those of the control group. [illegible], the pathological and histological

examination of these various organs of rats showed that there was no difference between these and those of the control group.

From the above, it is determined that the toxicity of the propionates and calcium propionate and sodium propionate, in particular, is almost non-existent.

On the basis of the aforementioned experimental results and in the light of various reliable reports, it is determined that these substances can be used in and added to certain foods.

In the above-described experiments, the pathological and histological examination was entrusted to Professor Yuichi Otaka, Director of the Pathological Department of this College. His name is especially mentioned here to show the gratitude of the authors.

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13) Hara et al.: Same as above, 20, 115 (1962)

14) Hara et al.: Same as above, 20, 175 (1962)

15) Hara et al.: Same as above, 20, 217 (1962)

16) Hara et al.: Same as above, 21, 111 (1963)

(Translated by Carl Demrick Associates,Inc/RO/t)

Table 1

C. P.	$(CH_3 \cdot CH_2 \cdot COO)_2Ca$
S. P.	$CH_3 \cdot CH_2 \cdot COONa$

Table 2

投与方法	C. P.	S. P.
静脈内交付	580 mg/kg	1,380 mg/kg
腹腔内交付	720 mg/kg	2,250 mg/kg
皮下交付	1,020 mg/kg	3,200 mg/kg
経口交付	3,340 mg/kg	5,100 mg/kg

Table 3-1

A 第 I 群	a 実験月日 (1961)		30/IX	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
	b 実験日数		1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
	c 飼育温度 (°C)		22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21
	d ラット体重 (g)	No. 1	81	83	90	99	102	t 交付開始相当日	105	107	112	118	121	127	127	133	136	140	141	146	148	148	152	152	154	156
		No. 2	82	83	94	94	93		99	100	106	108	111	117	118	121	127	130	130	134	138	140	143	140	150	149
		No. 3	76	76	85	92	101		100	103	109	111	112	118	120	123	124	126	131	137	139	141	145	144	151	152
		No. 4	74	72	84	88	95		100	102	109	110	110	115	116	114	114	116	118	124	126	127	129	131	137	136
		No. 5	73	70	82	86	88		88	91	96	99	99	105	103	110	112	113	114	116	118	121	124	119	127	126
		No. 6	68	64	76	84	86		89	90	98	103	104	112	113	113	115	116	120	124	124	125	133	133	135	136
		No. 7	67	67	77	84	90		96	98	104	106	105	113	114	113	118	117	122	126	128	130	131	130	138	134
		No. 8	62	64	74	82	84		87	96	93	96	97	103	108	112	113	113	117	121	122	127	130	129	131	133
		No. 9	67	68	75	80	88		93	93	99	103	100	113	115	115	117	117	118	125	127	130	132	132	136	138
		No. 10	64	68	76	79	79		83	88	92	94	98	102	117	107	110	111	114	119	121	122	128	128	131	137
		e 平均	71.4	71.5	81.3	86.8	90.6		94.0	95.8	101.8	104.8	105.7	112.5	115.1	116.1	118.6	119.9	122.5	127.2	129.1	131.1	134.7	133.8	139.0	139.7
	f 野菜交付量 (g)									n	ナッペツ 10							ナッペツ 10 o							ナッペツ 10 p	
	g 実験月日 (1961)		23	24	25	26	27	28	29	30	31	1/XI	2	3	4	5	6	7	8	9	10	11	12	13	14	
	h 実験日数		19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	
	i 飼育温度 (°C)		21	21	21	22	23	23	23	23	23	23	22	23	23	23	21	22	22	22	22	22	22	21	21	
	j ラット体重 (g)	No. 1	157	160	163	164	162	172	174	184	184	184	187	190	190	198	198	202	203	207	207	209	208	209	210	
		No. 2	151	152	155	156	156	168	168	173	174	175	178	180	184	187	187	192	192	202	197	203	204	205	205	
		No. 3	155	156	158	160	158	169	170	169	178	177	176	180	180	186	185	190	189	192	195	198	203	203	202	
		No. 4	136	139	142	143	142	151	153	155	160	161	166	171	174	176	179	180	180	184	190	192	190	191	192	
		No. 5	129	131	133	132	132	137	141	148	145	147	148	150	158	160	163	166	168	168	170	172	175	173	179	
		No. 6	138	137	141	140	138	141	146	152	159	157	158	160	163	166	168	170	173	175	177	177	177	179	181	
		No. 7	138	140	143	144	143	153	153	158	158	160	163	168	166	173	173	176	179	182	186	187	190	190	189	
		No. 8	137	137	143	143	143	153	155	159	161	164	166	170	174	176	176	179	181	186	187	187	190	188	188	
		No. 9	141	140	144	147	144	154	156	162	159	161	161	165	171	172	174	177	177	181	180	188	189	186	186	
		No. 10	133	135	141	142	142	148	153	155	153	155	159	157	159	166	164	168	170	178	178	179	181	180	199	
		k 平均	141.5	142.7	146.3	147.1	146.0	154.6	157.7	161.5	163.1	164.1	166.2	169.3	171.9	176.0	176.7	180.0	181.2	185.5	186.7	189.2	190.7	190.4	191.1	
	m 野菜交付量 (g)							ナッペツ 10 q							ナッペツ 10 r							ナッペツ 10 s				

Table 3-1

A 第 I 群

a 実験月日 (1961)		15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
b 実験日数		42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
c 飼育温度 (°C)		21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
d ラット体重 (g)	No. 1	218	216	218	227	231	228	232	240	239	234	251	249	243	251	257	253	252	254	257	260	262	263	266	264
	No. 2	210	213	210	224	226	224	228	228	231	228	241	239	237	242	247	241	238	250	252	242	240	238	239	242
	No. 3	208	208	208	222	223	223	226	230	232	232	241	243	241	250	253	251	247	255	258	263	269	268	275	273
	No. 4	197	199	197	210	215	216	218	222	223	225	233	230	234	241	244	239	237	244	247	247	248	253	254	240
	No. 5	183	182	184	200	205	200	202	203	206	206	214	215	213	217	215	210	207	215	215	212	218	219	220	216
	No. 6	183	187	185	197	205	206	206	212	211	216	222	221	221	225	225	223	216	225	230	229	231	235	237	232
	No. 7	189	198	199	209	217	211	218	220	226	231	240	239	241	245	253	251	247	255	254	258	265	260	264	260
	No. 8	190	196	199	209	212	210	214	208	209	200	206	205	206	209	205	199	194	193	149	190	201	199	204	203
	No. 9	189	194	194	211	215	215	213	218	220	220	225	227	231	235	236	248	232	239	239	238	242	244	248	248
	No. 10	180	189	190	194	199	200	202	203	206	208	212	212	213	214	217	213	212	216	221	217	223	223	222	220
e 平均		194.7	198.2	198.4	210.3	214.8	213.3	216.4	218.4	220.3	220.0	228.5	228.0	228.0	232.9	235.2	232.8	229.2	234.6	237.2	236.5	239.9	240.2	242.9	239.8
f 野菜交付量 (g)					キャベツ 10 n							キャベツ 10 o							キャベツ 10 p						

g 実験月日 (1961)		9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
h 実験日数		66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	
i 飼育温度 (°C)		22	22	22	22	22	22	22	23	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23	
j ラット体重 (g)	No. 1	265	267	267	270	267	278	277	277	273	272	278	270	271	268	268	273	264	266	267	265	261	262	261	
	No. 2	245	243	246	247	253	257	261	263	269	274	272	277	262	260	264	267	270	268	270	275	274	275	276	
	No. 3	276	278	280	284	278	287	289	290	285	287	293	287	289	285	290	291	283	291	284	289	286	290	288	
	No. 4	250	251	250	254	250	258	260	260	258	259	272	265	262	263	261	260	259	263	265	265	260	263	261	
	No. 5	217	213	213	213	210	214	217	214	213	213	212	210	214	218	225	230	234	243	242	247	245	246	247	
	No. 6	242	241	249	252	244	250	249	251	251	263	263	258	270	268	259	262	267	268	267	271	272	271	268	
	No. 7	272	272	274	275	273	283	285	286	281	286	292	286	288	292	295	296	298	300	301	306	302	307	305	
	No. 8	208	205	204	209	207	213	215	214	212	216	218	216	222	219	220	223	221	223	225	224	222	225	221	
	No. 9	248	248	249	251	250	260	258	259	253	257	263	255	262	262	264	268	263	266	269	274	268	273	273	
	No. 10	224	225	225	228	227	232	232	229	232	233	236	229	232	229	232	232	233	237	238	241	240	242	241	
k 平均		244.7	244.3	245.7	248.3	245.9	253.2	254.3	254.5	252.7	256.0	259.9	255.3	257.2	256.4	257.7	260.2	251.2	262.5	262.8	265.4	263.0	265.4	264.1	
m 野菜交付量 (g)		キャベツ 10 q							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

Table 3-2

第 A I 群

a 実験月日 (1962)		1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
b 実験日数		89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	204	105	106	107	108	109	110	111
c 飼育温度 (°C)		22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	22	21	22	22
d ラット体重 (g)	No. 1	262	263	260	264	265	265	264	263	268	268	264	267	263	265	259	254	261	260	261	262	265	257	258
	No. 2	289	293	297	300	303	301	303	304	306	303	303	305	303	309	303	303	310	311	311	315	316	313	314
	No. 3	289	290	288	289	298	288	289	287	290	284	289	294	293	302	290	288	293	296	297	297	296	292	299
	No. 4	260	261	259	262	265	265	263	264	268	262	266	272	269	274	268	265	272	267	264	266	270	263	273
	No. 5	249	247	248	251	253	250	255	257	262	257	258	258	259	265	263	266	271	271	267	265	271	265	265
	No. 6	269	271	269	272	275	275	275	277	278	274	272	272	273	275	269	266	277	270	269	272	277	273	269
	No. 7	305	308	308	312	308	310	312	311	313	326	324	324	317	324	325	318	325	330	330	334	337	330	335
	No. 8	224	223	225	224	225	230	238	229	233	237	234	235	237	240	239	235	241	238	241	241	244	234	244
	No. 9	269	278	275	274	273	274	275	277	283	283	284	283	286	296	279	282	286	285	289	292	295	290	296
	No. 10	240	242	239	245	243	247	247	242	247	247	246	250	249	253	247	245	249	248	250	247	252	249	251
e 平均		265.6	267.6	266.1	269.3	270.8	270.5	272.1	278.1	274.8	274.1	274.0	282.2	274.9	280.2	274.2	272.0	278.5	277.6	277.9	279.1	282.3	276.6	280.4
f 野菜交付量 (g)							キャベツ 10 n							キャベツ 10 o							キャベツ 10 p			
g 実験月日 (1692)		24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15
h 実験日数		112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
i 飼育温度 (°C)		22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
j ラット体重 (g)	No. 1	258	259	256	254	250	245	246	242	243	245	240	245	247	247	242	246	274	249	252	256	256	257	257
	No. 2	315	318	316	315	317	318	317	322	323	325	327	330	338	333	333	332	333	334	335	335	340	340	342
	No. 3	300	297	304	302	300	303	308	303	303	309	311	316	309	312	311	308	310	310	311	309	312	312	313
	No. 4	270	272	269	272	268	260	265	267	267	268	268	277	275	277	271	274	277	277	273	269	270	271	261
	No. 5	266	265	270	270	268	269	272	274	278	273	274	268	267	263	267	268	270	265	263	261	263	267	263
	No. 6	270	267	266	265	270	277	275	269	272	272	267	278	272	272	271	270	271	269	273	269	278	273	273
	No. 7	332	338	333	332	334	336	338	343	344	345	344	351	343	345	343	345	347	345	350	348	355	352	354
	No. 8	240	245	247	248	249	247	253	249	245	242	241	246	243	244	246	246	254	250	251	250	253	252	255
	No. 9	300	302	300	300	299	297	295	298	292	291	298	298	294	298	304	304	307	302	301	302	303	302	305
	No. 10	249	249	254	250	251	256	252	249	255	256	257	257	258	260	258	255	257	254	258	257	262	262	261
k 平均		280.0	281.2	281.5	280.8	280.4	280.8	282.1	281.6	282.2	282.7	282.7	286.6	284.6	285.1	284.6	284.8	287.3	285.5	286.7	285.6	289.2	288.6	283.4
m 野菜交付量 (g)					キャベツ 01 q							キャベツ 10 r							キャベツ 10 s					

Table 3-2

第 I 群 A.	a 実験月日 (1962)	16/II	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
	b 実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
	c 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
	d ラット体重 (g) No. 1	258	256	262	257	264	262	260	263	267	273	271	270	274	275	274	274	278	280	280	280	281	283	292
	No. 2	340	340	340	342	352	346	345	350	343	344	344	347	345	351	348	350	350	351	353	353	356	354	354
	No. 3	305	297	290	272	274	274	279	283	282	282	277	286	282	290	296	290	292	287	309	303	296	297	303
	No. 4	264	267	266	249	265	256	264	254	258	265	261	260	251	249	265	257	270	267	262	271	268	266	275
	No. 5	259	256	254	248	257	259	255	265	265	268	264	263	262	267	267	266	270	273	258	268	275	271	275
	No. 6	274	272	273	278	284	287	281	288	288	284	281	280	285	287	288	286	292	299	289	293	293	202	304
	No. 7	355	354	347	349	358	356	354	357	351	353	352	352	354	356	358	353	358	359	359	358	369	359	363
	No. 8	256	257	262	255	264	260	261	271	271	273	270	272	270	270	276	272	268	281	277	278	275	278	280
	No. 9	303	306	306	304	309	312	305	313	310	305	300	303	304	308	309	305	308	309	312	312	213	214	318
	No. 10	258	258	258	252	253	257	253	258	259	263	253	261	262	260	267	263	269	264	261	261	233	262	270
	e 平均	287.2	286.3	285.8	280.6	288.0	286.9	285.7	290.2	289.4	291.0	287.3	289.4	288.9	291.9	294.8	291.6	295.5	297.0	296.0	297.7	298.0	298.6	302.9
	f 野菜交付量 (g)		キャベツ 10 n							キャベツ 10 o							キャベツ 10 p							キャベツ 10 q
	g 実験月日 (1962)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
	h 実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
	i 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
	j ラット体重 (g) No. 1	291	290	290	293	289	293	295	298	300	300	303	297	298	301	302	306	306	305	305	305	309	307	304
	No. 2	364	356	356	357	358	361	358	362	360	363	367	366	367	367	368	375	373	374	374	372	372	376	378
	No. 3	313	298	300	301	307	310	311	312	316	315	320	321	324	321	322	328	328	330	330	330	330	331	332
	No. 4	284	275	286	289	277	272	283	280	266	268	261	263	280	285	284	282	273	284	285	286	289	285	286
	No. 5	279	272	276	276	275	277	280	282	294	293	286	286	289	290	290	301	298	298	295	297	299	298	300
	No. 6	307	286	292	294	295	295	296	302	302	306	303	305	306	304	304	314	310	313	313	314	317	311	324
	No. 7	364	345	352	361	361	361	362	364	371	371	367	367	368	365	370	383	380	379	379	377	376	376	389
	No. 8	284	284	288	288	284	288	288	292	298	300	303	301	298	304	303	306	308	309	309	307	309	309	312
	No. 9	316	317	319	326	326	325	323	318	327	330	338	332	330	331	329	336	335	332	337	337	336	335	339
	No. 10	274	276	276	282	287	283	280	282	282	284	284	283	287	283	286	286	281	289	289	287	288	289	295
	k 平均	307.6	299.9	303.5	306.7	305.9	306.5	307.6	309.2	311.6	313.0	313.2	312.1	314.7	315.1	315.8	321.7	319.6	321.3	321.6	321.2	322.5	321.7	325.9
	m 野菜交付量 (g)							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

Table 4-1

A 第 II 群

a 実験月日 (1961)		30/III	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22				
b 実験日数		1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18				
c 飼育温度 (°C)		22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21				
d ラット体重 (g)	No. 1	86	87	98	103	104	t 交付開始	108	109	114	115	116	119	122	123	126	126	128	132	137	138	143	138	143	144				
	No. 2	79	79	90	96	97		102	103	106	109	113	113	119	121	126	130	132	134	139	140	144	143	153	153				
	No. 3	76	78	88	93	98		103	101	112	113	115	115	115	114	116	117	118	122	126	127	130	129	132	136				
	No. 4	77	75	85	89	100		102	101	107	107	107	111	112	116	120	119	121	126	126	129	132	134	140	141				
	No. 5	77	73	82	87	97		97	97	104	105	104	108	110	112	114	117	120	121	127	127	133	132	138	138				
	No. 6	67	67	77	85	81		86	85	91	94	96	99	101	103	105	107	109	113	118	118	121	117	121	123				
	No. 7	68	68	78	84	90		93	95	101	106	109	111	113	113	115	116	116	121	122	123	123	123	126	127				
	No. 8	70	69	77	82	87		90	93	101	102	107	110	114	113	115	117	120	119	123	123	125	125	127	129				
	No. 9	69	72	80	80	84		92	93	102	106	111	113	114	116	119	121	124	127	129	131	134	133	131	135				
	No. 10	66	70	73	79	83		88	91	97	101	104	105	106	107	108	110	113	115	118	120	124	124	132	131				
e 平均		73.5	73.8	82.8	87.8	92.1		96.1	96.8	103.5	105.8	108.2	110.4	112.6	113.8	116.4	118.0	120.1	123.0	126.5	127.6	130.9	129.8	131.6	135.7				
f 野菜交付量 (g)										キャベツ 10 h							キャベツ 10 o							キャベツ 10 p				
g 実験月日 (1961)		23	24	25	26	27	28	29	30	31	1/IX	2	3	4	5	6	7	8	9	10	11	12	13	14					
h 実験日数		19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41					
i 飼育温度 (°C)		21	21	21	22	23	23	23	23	23	23	22	23	23	23	21	22	22	22	22	22	22	21	21					
j ラット体重 (g)	No. 1	144	145	149	149	148	157	158	163	163	166	165	170	174	180	177	180	186	187	186	188	192	192	193					
	No. 2	153	155	156	159	156	166	170	172	171	173	174	178	180	176	184	184	188	189	192	193	197	196	198					
	No. 3	137	138	141	141	141	150	154	156	162	161	162	163	165	169	166	166	173	172	175	175	178	175	177					
	No. 4	143	145	148	149	149	157	160	163	160	164	166	167	172	175	173	173	181	181	183	186	189	188	189					
	No. 5	139	141	145	145	147	155	158	164	163	166	169	171	175	180	180	187	191	189	191	194	195	195	196					
	No. 6	125	125	127	132	130	136	140	139	138	143	145	148	150	153	152	156	166	158	162	164	166	167	170					
	No. 7	128	130	136	138	136	142	146	152	155	153	155	156	159	156	170	169	168	168	168	174	176	176	178					
	No. 8	130	131	132	138	134	143	148	152	152	153	155	158	160	165	167	172	176	177	179	185	186	189	188					
	No. 9	134	136	136	137	135	147	147	155	153	156	156	161	165	169	170	173	177	176	177	183	187	188	190					
	No. 10	134	137	139	141	139	149	150	161	156	157	159	164	173	173	171	171	176	176	178	181	183	183	184					
k 平均		136.7	138.3	140.9	142.9	141.5	150.2	153.1	157.7	157.3	159.2	160.6	163.6	167.2	169.6	171.0	173.1	178.2	177.3	179.1	182.3	184.9	184.9	186.3					
m 野菜交付量 (g)							キャベツ 10 q							キャベツ 10 r							キャベツ 10 s							

Table 4-1

第II群																									
a 実験月日 (1961)		15/XII	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
b 実験日数		42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
c 飼育温度 (°C)		22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
d ラット体重 (g)	No. 1	197	199	198	211	215	213	218	220	220	218	227	228	225	231	233	231	229	238	243	240	239	243	246	243
	No. 2	204	205	204	216	220	218	222	223	226	224	236	237	228	240	242	243	234	245	248	244	247	250	257	253
	No. 3	184	184	184	200	206	205	205	210	211	208	215	225	219	221	226	223	222	232	236	239	238	241	247	246
	No. 4	195	194	194	208	214	213	212	218	219	220	231	229	227	233	237	237	234	240	247	242	243	246	250	248
	No. 5	199	203	202	217	221	221	224	225	228	227	235	236	239	240	242	239	236	247	257	248	250	253	258	253
	No. 6	174	174	174	188	194	191	202	208	203	204	210	215	218	216	221	222	215	228	232	230	232	235	244	241
	No. 7	184	184	185	195	208	205	210	215	213	216	222	224	225	225	230	234	232	236	238	237	243	241	247	247
	No. 8	193	193	198	202	207	208	206	208	209	208	211	213	211	215	216	218	218	220	223	219	221	223	229	223
	No. 9	194	194	194	207	214	206	218	222	226	224	238	212	242	247	250	248	247	257	261	258	259	256	267	261
	No. 10	193	194	195	206	213	213	213	218	217	218	229	228	229	228	236	242	233	243	249	247	247	250	257	252
e 平均		191.7	192.4	192.8	205.0	211.2	209.3	213.0	216.7	217.2	216.7	225.4	227.7	226.3	229.6	233.3	233.7	230.0	238.6	243.4	240.4	241.9	243.8	250.2	246.7
f 野菜交付量 (g)					キャベツ 10 h							キャベツ 10 o							キャベツ 10 p						
g 実験月日 (1961)		9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
h 実験日数		66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	
i 飼育温度 (°C)		22	22	22	22	22	22	22	23	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23	
j ラット体重 (g)	No. 1	251	248	262	262	255	260	265	265	264	272	273	268	271	269	262	270	275	276	275	278	270	285	276	
	No. 2	258	260	273	274	262	275	279	273	269	271	279	272	275	274	274	280	283	283	281	287	280	292	284	
	No. 3	252	252	259	262	261	264	271	265	268	265	272	267	269	267	255	272	278	276	270	277	270	284	282	
	No. 4	253	252	263	265	259	264	268	269	269	268	271	273	273	272	274	273	277	282	274	281	274	287	281	
	No. 5	259	259	271	279	273	274	280	281	280	278	282	286	283	280	283	284	287	291	289	295	288	297	299	
	No. 6	244	243	252	252	250	263	259	263	258	258	262	258	260	260	262	262	262	272	268	272	274	279	273	
	No. 7	245	245	255	253	245	263	264	260	261	263	267	263	268	270	274	270	275	273	277	279	272	276	275	
	No. 8	228	230	232	237	229	243	240	245	247	247	251	253	251	253	255	258	257	258	257	263	258	262	261	
	No. 9	269	267	283	287	280	290	291	294	295	298	305	302	301	299	300	304	302	305	309	310	305	322	318	
	No. 10	254	255	270	270	264	273	277	269	275	281	278	278	276	272	274	274	282	285	284	289	285	299	297	
k 平均		251.3	251.1	262.0	264.1	258.0	266.9	269.4	268.4	268.6	270.1	274.0	272.0	272.7	271.6	271.3	274.7	277.8	280.1	278.4	283.1	277.6	288.3	284.6	
m 野菜交付量 (g)		キャベツ 10 q							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

Table 4-2

第 II 群

a 実験月日 (1961)		1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
b 実験日数		89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
c 飼育温度 (°C)		22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22
d ラット体重 (g)	No. 1	287	275	285	288	286	286	286	280	283	279	273	287	276	280	265	268	277	274	272	272	275	270	274
	No. 2	298	298	303	306	302	310	311	312	310	308	307	308	309	311	297	303	308	302	304	306	308	301	300
	No. 3	285	285	283	282	282	282	281	280	280	282	281	286	286	298	266	276	280	277	277	278	279	271	278
	No. 4	290	291	292	295	294	299	295	300	304	300	296	300	296	299	285	291	290	291	290	290	297	292	294
	No. 5	297	299	298	298	301	295	302	303	305	307	308	302	302	307	288	297	302	300	298	300	302	302	304
	No. 6	276	282	278	278	283	277	283	284	287	284	286	290	287	289	265	276	284	282	283	280	287	281	286
	No. 7	276	277	278	277	282	282	282	283	286	286	287	290	288	291	266	279	289	290	291	292	288	289	289
	No. 8	262	265	265	267	270	274	271	274	279	279	280	284	286	284	266	277	283	281	288	288	287	284	285
	No. 9	322	324	330	334	334	338	341	335	333	328	328	334	330	329	310	321	320	321	322	327	330	326	333
	No. 10	305	305	302	304	306	306	309	308	306	305	306	306	304	306	289	301	301	301	298	297	298	298	297
	e 平均	289.8	290.1	291.4	292.9	294.0	294.9	296.1	295.9	297.3	295.8	295.2	298.7	296.4	299.4	279.6	288.9	293.4	292.2	292.3	293.0	299.4	291.7	294.0
f 野菜交付量 (g)							キャベツ 10 n							キャベツ 10 o							キャベツ 10 p			

第 II 群

g 実験月日 (1961)		24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15
h 実験日数		112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
i 飼育温度 (°C)		22	21	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22
j ラット体重 (g)	No. 1	272	272	272	270	275	270	275	276	270	271	270	280	274	274	273	274	273	275	277	268	276	278	275
	No. 2	304	305	303	305	304	311	309	306	306	306	306	307	311	309	311	311	314	316	315	315	316	315	315
	No. 3	273	274	273	279	279	275	282	276	275	277	276	281	272	273	280	271	268	267	263	259	267	262	265
	No. 4	292	290	285	295	292	305	300	300	300	298	300	308	297	300	300	298	302	301	303	301	302	301	302
	No. 5	302	302	303	303	300	302	302	302	302	304	302	304	303	305	306	308	312	310	314	316	319	315	319
	No. 6	283	287	287	280	282	291	291	289	293	297	292	302	293	295	294	292	294	297	299	298	300	301	305
	No. 7	287	289	291	290	294	298	296	302	307	310	310	314	311	313	310	312	315	317	317	319	321	321	324
	No. 8	285	286	288	287	293	300	304	303	302	301	305	307	303	300	302	300	305	306	309	309	311	311	310
	No. 9	330	330	333	332	333	336	333	334	332	335	335	342	339	337	337	337	340	338	342	343	340	344	345
	No. 10	295	295	296	291	291	306	298	297	295	295	295	298	295	295	294	292	293	294	297	295	296	295	300
	k 平均	292.3	293.0	293.1	293.2	294.3	299.4	300.0	298.5	298.2	299.7	299.1	304.3	299.8	300.1	300.7	299.5	301.6	302.1	303.6	302.3	304.8	301.3	306.0
m 野菜交付量 (g)					キャベツ 10 q							キャベツ 10 r							キャベツ 10 s					

Table 4-2

第II群																								
a 実験月日 (1961)	16/II	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
b 実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
c 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
d ラット体重 (g) No. 1	269	270	272	265	267	271	271	276	270	271	272	276	274	277	277	273	276	279	282	279	278	280	278
No. 2	311	312	312	312	316	319	316	319	319	319	310	316	315	320	320	313	320	310	325	320	336	325	325
No. 3	267	266	264	258	264	267	267	272	272	273	265	269	264	266	267	262	268	267	270	273	273	275	275
No. 4	302	298	296	297	300	301	299	301	303	305	300	302	296	302	304	297	300	292	305	303	305	306	304
No. 5	316	313	300	317	321	319	320	321	321	323	317	313	316	316	318	313	314	324	321	320	323	322	321
No. 6	301	310	304	308	310	309	308	310	304	299	295	295	292	295	294	295	300	295	305	305	301	305	303
No. 7	324	315	324	330	326	331	337	339	339	337	340	340	340	347	346	342	342	329	343	346	342	346	341
No. 8	305	307	312	312	315	315	316	317	315	315	323	322	317	321	322	317	324	332	331	328	331	331	341
No. 6	344	345	350	348	352	346	350	351	351	354	350	351	350	355	356	350	349	357	355	354	351	352	350
No. 10	299	294	302	304	305	303	302	304	304	305	302	303	304	304	307	304	304	313	308	308	306	310	308
e 平均	303.8	303.0	303.6	305.1	307.6	308.1	308.6	311.0	309.8	310.1	307.4	308.7	306.8	310.3	311.1	306.6	309.7	309.8	314.5	318.6	314.6	315.2	314.6
f 野菜交付量 (g)		キャベツ 10 n							キャベツ 10 o							キャベツ 10 p							キャベツ 10 q
g 実験月日 (1961)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
h 実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
i 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
j ラット体重 (g) No. 1	284	268	276	277	279	280	282	286	289	290	291	290	288	297	292	297	296	303	302	302	306	304	315
No. 2	329	312	318	321	318	322	320	328	324	326	329	332	323	330	330	335	335	332	333	332	333	334	340
No. 3	287	283	282	286	285	286	283	292	298	296	296	295	292	302	301	306	305	306	305	301	303	302	311
No. 4	310	311	311	310	309	312	312	318	326	321	321	322	316	320	318	321	321	322	322	322	319	321	336
No. 5	330	327	330	328	328	328	328	334	338	340	336	336	333	332	334	340	335	339	335	335	338	338	341
No. 6	309	308	308	312	313	314	316	311	318	318	316	316	318	320	322	324	323	324	322	327	331	328	328
No. 7	346	346	342	346	347	347	347	348	352	354	352	354	355	354	355	358	358	358	363	362	360	365	364
No. 8	337	319	339	343	347	346	343	346	350	348	346	344	343	344	347	358	355	358	352	355	352	354	353
No. 9	356	357	355	356	354	359	354	360	372	366	368	367	367	363	367	382	373	368	370	372	373	372	371
No. 10	314	313	312	318	314	313	312	318	322	320	320	320	318	318	321	325	320	321	323	322	326	323	329
k 平均	320.2	314.4	317.3	319.7	319.4	321.7	319.7	324.1	328.9	327.9	327.5	327.6	325.3	328.0	328.7	334.6	332.1	333.1	332.7	333.0	334.1	334.1	338.8
m 野菜交付量 (g)							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

Table 5-1

第III群																												
a 実験月日 (1961)		30/IX	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
b 実験日数		1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
c 飼育温度 (°C)		22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21
d ラット体重 (g)	No. 1	90	88	100	105	112	t 交付開始	111	110	114	116	116	116	118	120	122	122	124	127	127	128	132	131	132	134
	No. 2	77	75	86	96	98		101	102	104	103	98	96	93	95	95	102	95	107	112	119	118	119	120	120
	No. 3	78	76	87	93	94		96	100	99	101	98	100	102	103	107	108	108	107	109	111	113	114	115	118
	No. 4	73	74	85	91	96		102	96	106	107	107	106	111	113	116	115	120	117	120	122	125	124	131	127
	No. 5	75	75	82	87	88		92	90	93	101	101	105	106	112	119	119	120	121	123	126	130	131	133	134
	No. 6	70	70	78	85	93		92	95	97	100	103	103	105	110	111	112	112	112	117	116	119	117	120	121
	No. 7	70	69	79	84	83		89	82	88	88	87	84	86	96	97	98	99	99	102	112	119	117	121	124
	No. 8	71	70	79	83	93		90	92	94	97	98	101	102	103	109	110	114	113	116	117	120	120	127	128
	No. 9	67	69	77	81	88		84	92	94	98	98	102	104	106	111	114	116	116	120	121	123	126	124	126
	No. 10	62	65	74	88	85		88	88	90	97	95	101	103	104	109	112	114	114	117	120	123	121	130	129
	e 平均	73.3	73.1	82.7	89.3	93.0		94.5	94.7	97.9	100.8	100.1	101.4	103.0	106.2	109.6	111.2	112.4	113.3	116.3	119.2	122.2	122.0	125.3	126.1
f 野菜交付量 (g)										キャベツ 10 n							キャベツ 10 o							キャベツ 10 p	
g 実験月日 (1961)		23	24	25	26	27	28	29	30	31	1/XI	2	3	4	5	6	7	8	9	10	11	12	13	14	
h 実験日数		19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	
i 飼育温度 (°C)		21	21	21	22	23	23	23	23	23	23	23	23	23	23	21	22	22	22	22	22	22	21	21	
j ラット体重 (g)	No. 1	138	137	138	141	139	151	151	158	149	152	152	153	158	161	166	163	166	167	169	174	175	176	176	
	No. 2	122	122	123	124	124	133	136	146	139	136	139	141	140	148	152	150	149	151	152	156	155	156	160	
	No. 3	116	115	118	120	128	134	141	142	143	145	145	148	150	152	156	150	160	160	163	164	166	165	169	
	No. 4	131	129	134	134	131	145	146	160	155	154	156	162	169	170	177	175	177	178	181	187	187	189	190	
	No. 5	133	135	135	135	138	141	147	142	150	150	153	152	154	156	160	158	161	163	167	170	168	171	170	
	No. 6	126	127	129	137	131	139	144	147	146	146	150	154	154	155	160	161	163	165	167	176	176	177	177	
	No. 7	122	122	123	130	130	136	141	138	143	142	148	146	150	150	150	153	153	156	156	146	153	153	151	
	No. 8	129	130	133	133	131	136	141	145	141	146	145	148	148	152	155	156	159	161	161	164	166	169	167	
	No. 9	126	129	130	133	133	141	145	153	150	148	152	156	163	165	167	169	169	170	172	176	178	179	181	
	No. 10	132	131	137	138	138	144	147	157	154	154	155	158	163	165	168	166	168	170	173	176	178	165	172	
	k 平均	127.5	127.7	129.5	132.5	132.3	140.0	143.9	148.8	147.0	147.3	149.5	151.8	154.9	157.4	161.1	160.8	162.5	164.1	166.1	168.9	170.2	169.8	171.1	
m 野菜交付量 (g)							キャベツ 10 q							キャベツ 10 r							キャベツ 10 s				

Table 5-1

a 実験月日 (1961)		15/XII	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
b 実験日数		42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
c 飼育温度 (°C)		21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
d ラット体重 (g)	No. 1	182	182	184	192	199	197	202	201	203	207	211	216	216	217	220	219	208	226	226	225	228	230	231	231
	No. 2	163	166	170	176	180	182	183	187	187	189	193	195	191	196	201	198	195	201	211	211	209	209	212	213
	No. 3	171	170	176	186	195	196	199	203	203	200	207	210	200	203	206	208	204	213	219	215	219	218	221	224
	No. 4	195	198	201	209	213	217	217	223	226	226	233	233	228	235	238	234	230	240	255	244	248	247	252	248
	No. 5	173	168	168	170	174	176	171	172	174	196	177	182	180	183	184	186	184	186	191	192	190	190	193	196
	No. 6	183	184	183	192	198	198	200	206	208	211	220	222	217	222	228	225	225	233	240	240	241	240	244	241
	No. 7	155	157	153	156	166	168	170	182	178	183	190	192	187	192	192	192	188	200	197	202	203	203	208	206
	No. 8	172	171	173	177	177	166	180	182	183	181	188	191	188	193	193	195	195	195	196	195	200	198	200	199
	No. 9	185	187	188	198	200	205	207	211	214	216	221	231	220	226	230	232	229	235	241	242	242	244	247	247
	No. 10	176	177	179	186	194	193	195	199	205	207	210	214	215	221	227	226	222	224	227	226	229	228	231	225
	e 平均	175.5	176.0	177.5	184.2	189.6	189.8	192.4	196.6	198.1	200.4	205.0	208.6	204.2	208.8	212.2	211.5	208.0	215.3	220.3	219.2	220.9	220.6	224.5	223.0
f 野菜交付量 (g)				n	キャベツ 10							キャベツ 10 o							キャベツ 10 p						
g 実験月日 (1961)		9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
h 実験日数		66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	
i 飼育温度 (°C)		22	22	23	22	22	22	22	23	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23	
j ラット体重 (g)	No. 1	241	240	243	249	245	244	250	232	250	246	248	248	254	239	238	233	237	241	243	248	239	243	240	
	No. 2	219	216	229	238	227	236	238	237	241	243	245	241	247	244	246	248	252	261	267	274	274	281	280	
	No. 3	226	231	231	238	235	234	241	242	237	235	239	237	239	239	243	240	250	247	257	262	272	267	262	
	No. 4	253	255	272	272	267	274	275	275	283	281	286	283	286	280	285	290	292	302	303	307	305	320	317	
	No. 5	195	200	200	207	208	203	210	212	209	213	214	211	219	213	219	220	215	222	225	227	225	230	229	
	No. 6	250	249	268	264	258	264	270	270	270	272	275	274	273	276	280	280	286	286	288	294	290	313	307	
	No. 7	213	209	222	227	218	227	227	229	236	232	233	235	238	236	240	239	244	244	243	254	252	262	255	
	No. 8	202	200	200	205	207	209	215	213	213	215	218	216	221	218	222	222	223	225	227	232	231	232	233	
	No. 9	249	245	258	263	251	255	259	264	264	258	264	261	260	263	265	267	269	272	268	274	272	273	270	
	No. 10	232	232	238	241	239	242	243	244	243	247	249	247	247	244	245	244	245	241	244	246	245	247	245	
	k 平均	228.0	227.7	236.5	240.4	235.5	238.8	242.8	241.8	244.6	244.2	247.1	245.3	248.4	245.2	248.3	248.3	251.3	254.1	256.5	261.8	260.5	266.8	263.8	
m 野菜交付量 (g)		キャベツ 10 q							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

第 III 群

A.

Table 5-2

第 A III 群	a 実験月日 (1962)	1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	b 実験日数	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
	c 飼育温度 (°C)	22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22
	d ラット体重 (g) No. 1	238	245	243	240	240	250	243	242	249	248	245	248	247	255	251	258	261	258	263	260	261	271	262
	No. 2	280	287	291	285	290	292	297	298	296	294	290	293	292	295	294	305	303	303	303	307	309	317	311
	No. 3	268	258	269	266	267	270	274	278	272	270	267	269	270	280	283	275	277	278	275	279	283	287	290
	No. 4	323	324	325	326	328	335	335	336	331	329	323	328	323	329	324	337	333	332	329	331	337	333	335
	No. 5	227	230	232	231	231	231	233	234	238	240	240	241	246	245	240	242	242	248	245	251	249	251	257
	No. 6	311	318	323	326	323	328	322	330	328	325	320	323	323	323	323	331	329	324	323	327	328	329	331
	No. 7	260	256	270	269	268	271	273	270	274	276	269	273	269	277	268	273	270	275	275	276	274	275	281
	No. 8	230	235	238	239	239	241	243	245	247	250	252	253	256	260	256	257	263	262	264	267	269	261	264
	No. 9	270	273	273	272	270	270	273	273	277	274	275	275	275	280	275	283	284	286	284	289	285	286	286
	No. 10	249	242	248	245	246	246	245	245	254	251	252	254	255	263	251	258	260	259	262	263	264	260	262
	e 平均	265.6	266.8	271.2	269.9	270.2	273.4	273.3	274.5	276.6	275.7	273.3	275.7	275.6	280.7	276.3	281.9	282.2	282.5	280.3	285.0	285.7	287.3	289.9
	f 野菜交付量 (g)						ナッペ 10 n							ナッペ 10 o							ナッペ 10 p			
第 III 群	g 実験月日 (1962)	24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	h 実験日数	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
	i 飼育温度 (°C)	22	21	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22
	j ラット体重 (g) No. 1	261	264	265	262	265	276	267	271	270	269	270	275	276	273	279	274	282	275	279	285	282	283	283
	No. 2	311	312	312	313	314	329	324	322	324	327	325	336	330	327	325	321	323	321	321	332	330	330	330
	No. 3	283	289	290	286	291	292	294	293	300	304	303	313	301	300	305	301	305	303	310	307	308	310	312
	No. 4	331	333	333	333	333	339	338	340	343	338	340	345	342	340	338	337	340	336	339	342	343	342	342
	No. 5	254	254	259	261	259	262	259	264	265	262	268	270	290	271	267	265	270	271	266	273	274	273	276
	No. 6	328	329	329	327	320	333	337	331	330	331	330	331	328	330	330	334	334	330	331	339	340	337	337
	No. 7	296	286	287	284	285	294	295	290	294	293	288	300	287	291	287	295	295	289	293	298	299	297	303
	No. 8	264	266	265	267	272	274	275	272	277	280	278	281	275	282	280	279	281	285	291	291	289	291	295
	No. 9	284	288	293	292	296	292	296	299	288	297	292	294	290	287	290	291	289	293	297	298	300	299	303
	No. 10	258	263	262	264	262	270	273	274	277	274	275	281	277	278	278	274	280	280	283	281	284	286	287
	k 平均	285.0	288.4	289.5	288.9	289.7	296.1	295.8	295.6	296.8	297.5	296.9	302.6	299.6	297.8	297.9	297.1	300.2	298.3	301.0	301.6	304.9	304.8	306.8
	m 野菜交付量 (g)				ナッペ 10 q							ナッペ 10 r							ナッペ 10 s					

Table 5-2

a 実験月日 (1962)	16/II	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
b 実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
c 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
d ラット体重 (g) No. 1	284	279	288	295	290	290	290	292	290	291	296	292	294	296	294	294	294	309	302	300	301	300	300
No. 2	329	327	329	332	332	332	332	336	338	341	343	342	342	344	346	339	339	349	342	343	339	343	340
No. 3	310	308	310	322	318	316	316	321	320	316	322	324	326	326	329	325	327	342	337	337	339	337	335
No. 4	341	340	340	350	352	350	348	356	355	353	353	353	353	356	356	354	356	368	360	362	354	354	355
No. 5	274	275	281	277	280	282	283	284	284	384	291	291	286	293	290	291	292	301	299	300	297	300	297
No. 6	339	339	339	341	339	338	340	346	346	346	345	342	344	345	346	347	348	353	353	356	352	351	357
No. 7	295	300	300	305	310	310	310	316	315	314	308	313	312	312	317	315	314	300	313	319	312	317	313
No. 8	295	294	298	297	302	300	302	303	302	303	306	311	310	315	319	314	314	318	316	316	313	316	317
No. 9	301	302	301	304	306	306	307	312	313	313	311	313	309	313	311	310	314	314	314	315	310	319	318
No. 10	289	285	287	287	290	294	292	295	295	295	296	296	296	299	301	296	298	301	298	299	300	300	298
e 平均	305.7	304.9	307.3	311.0	311.9	311.8	312.0	316.1	315.8	315.6	317.1	317.7	317.2	319.9	320.9	319.5	319.6	325.5	323.4	324.7	321.7	323.7	323.3
f 野菜交付量 (g)		キャベツ 10 n							キャベツ 10 o							キャベツ 10 p							キャベツ 10 q
g 実験月日 (1962)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
h 実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
i 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
j ラット体重 (g) No. 1	302	304	300	300	301	302	301	310	315	313	315	316	311	314	315	318	318	320	317	317	317	319	323
No. 2	342	350	344	342	344	344	343	346	352	348	350	355	354	352	353	358	355	354	354	350	355	353	358
No. 3	336	335	340	339	337	338	338	342	350	350	346	344	348	327	346	362	350	351	359	350	352	354	353
No. 4	364	365	360	360	358	362	358	359	377	374	368	371	371	369	371	378	369	375	371	373	371	374	379
No. 5	300	302	300	300	305	302	303	309	311	311	312	311	313	309	306	318	318	318	325	316	317	318	320
No. 6	354	357	351	357	355	357	356	362	381	374	371	369	369	370	365	372	370	370	371	371	371	371	376
No. 7	310	320	319	327	322	323	325	322	326	332	329	322	334	336	335	338	334	335	339	339	341	337	338
No. 8	316	317	320	321	319	322	328	320	326	330	330	331	332	337	331	337	334	340	339	333	337	332	332
No. 9	322	318	308	324	322	324	326	323	325	329	330	325	326	330	328	328	332	332	332	329	329	329	337
No. 10	303	303	298	301	303	303	302	300	301	303	304	303	302	307	306	302	305	308	304	305	305	306	306
k 平均	324.9	327.1	324.0	327.1	326.1	327.7	328.0	329.3	336.4	336.4	335.5	334.7	336.0	335.1	335.6	341.1	338.5	340.3	340.1	338.3	339.5	339.3	342.2
m 野菜交付量 (g)							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

第III群 A

Table 6-1

a 実験月日 (1961)		30/IX	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
b 実験日数		1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
c 飼育温度 (°C)		22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21
d ラット体重 (g)	No. 1	88	86	98	106	107	t 交付開始	107	110	112	115	116	119	120	121	128	129	130	133	134	137	141	141	143	146
	No. 2	85	81	91	97	98		98	100	101	102	107	108	104	105	111	112	116	117	120	120	125	124	122	132
	No. 3	77	77	89	94	99		94	91	91	94	95	101	95	99	104	108	110	113	115	117	121	121	120	124
	No. 4	76	76	88	91	97		92	94	99	97	101	104	102	104	110	111	112	114	118	121	125	123	124	131
	No. 5	71	71	80	88	88		89	89	88	87	93	94	93	97	103	107	107	112	115	117	121	119	126	126
	No. 6	73	73	82	86	90		86	87	94	88	95	96	91	93	96	100	102	105	107	109	111	110	112	114
	No. 7	67	69	79	84	91		89	91	95	96	102	105	105	114	118	119	121	126	127	130	132	132	133	136
	No. 8	66	64	76	83	85		84	83	83	81	87	91	86	91	95	97	100	103	106	109	110	114	117	117
	No. 9	67	67	76	82	83		83	84	88	86	82	92	93	95	97	98	99	100	104	103	99	113	109	111
	No. 10	66	63	73	80	84		82	85	86	89	90	93	92	93	100	102	104	107	109	112	116	115	117	118
	e 平均	73.6	72.7	83.2	89.1	92.2		90.4	91.4	93.7	93.5	96.8	100.3	98.1	101.2	106.2	108.3	100.1	113.0	115.5	117.5	120.1	121.2	122.3	125.5
f 野菜交付量 (g)										キャベツ 10 n							キャベツ 10 o							キャベツ 10 p	
g 実験月日 (1961)		23	24	25	26	27	28	29	30	31	1/IX	2	3	4	5	6	7	8	9	10	11	12	13	14	
h 実験日数		19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	
i 飼育温度 (°C)		21	21	21	22	23	23	23	23	73	23	22	23	23	23	21	22	22	22	22	22	22	21	21	
j ラット体重 (g)	No. 1	148	148	151	154	153	160	164	171	169	169	171	174	180	179	183	183	186	187	186	194	192	197	200	
	No. 2	132	133	137	137	137	141	145	152	150	150	152	153	154	160	158	160	164	164	163	168	168	175	176	
	No. 3	128	127	132	134	132	139	140	141	148	147	148	153	155	158	164	160	161	161	163	171	173	175	175	
	No. 4	127	132	133	138	136	140	150	156	151	152	154	157	151	166	166	169	168	165	169	173	170	179	176	
	No. 5	128	128	132	132	132	140	147	154	149	150	151	155	157	164	163	165	169	170	170	177	178	184	183	
	No. 6	116	118	120	124	122	127	132	135	138	140	140	143	145	146	148	146	152	154	156	159	161	161	163	
	No. 7	136	137	140	140	140	149	151	156	153	153	156	163	162	160	162	165	171	171	172	178	180	193	190	
	No. 8	120	122	133	140	141	146	150	154	146	150	147	153	155	158	158	165	165	165	163	169	171	176	176	
	No. 9	113	112	112	120	124	136	140	134	136	144	144	141	147	147	148	148	153	153	151	155	159	159	160	
	No. 10	118	118	121	123	123	129	133	136	132	134	136	139	145	145	147	146	149	150	150	157	156	163	161	
	k 平均	126.6	127.5	131.1	134.2	134.0	147.0	145.2	148.9	147.2	148.9	149.9	153.1	155.1	158.3	159.7	160.7	163.8	164.0	164.6	170.1	170.8	176.2	176.0	
m 野菜交付量 (g)							キャベツ 10 q							キャベツ 10 r							キャベツ 10 s				

第 IV 群

A

Table 6-2

	a 実験月日 (1961)	15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
	b 実験日数	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
	c 飼育温度 (°C)	21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
第IV群	d ラット体重 (g) No. 1	200	204	206	211	222	224	226	231	236	242	249	243	244	249	249	248	244	247	249	255	259	261	266	264
	No. 2	178	179	180	182	195	196	197	201	201	211	218	226	223	225	223	229	222	234	232	237	237	238	242	236
	No. 3	175	179	181	185	197	196	196	202	203	213	220	219	215	217	215	216	208	221	224	225	225	228	234	238
	No. 4	180	178	181	186	196	197	200	201	201	204	209	212	211	214	217	218	213	220	220	215	218	218	219	215
	No. 5	188	187	186	193	197	205	201	205	204	204	215	216	218	217	216	222	209	215	222	218	223	221	225	223
	No. 6	168	169	170	171	170	171	171	173	178	174	179	178	178	181	184	184	183	185	181	186	187	187	185	192
	No. 7	193	197	198	206	211	214	213	224	223	235	243	243	243	243	244	243	239	247	247	250	256	251	256	256
	No. 8	179	185	184	190	198	200	103	207	206	212	224	231	223	225	229	227	219	229	232	238	236	235	238	238
	No. 9	160	165	164	165	167	169	172	173	174	171	175	175	175	178	178	178	178	176	182	185	186	186	185	189
	No. 10	163	165	164	170	173	178	183	183	182	191	197	199	195	196	197	197	194	200	208	208	211	210	212	211
	e 平均	178.4	180.8	181.4	185.9	192.6	195.0	196.2	200.0	200.8	205.7	212.9	214.2	212.5	215.5	215.2	216.2	210.9	217.4	219.7	221.2	223.8	223.5	226.6	226.2
	f 野菜交付量 (g)				キャベツ 10 n							キャベツ 10 o							キャベツ 10 p						
A群	g 実験月日 (1961)	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
	h 実験日数	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	
	i 飼育温度 (°C)	22	22	22	22	22	21	22	22	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23	
	j ラット体重 (g) No. 1	269	275	282	285	275	278	291	286	285	288	287	284	289	283	290	287	289	288	291	298	301	306	306	
	No. 2	245	245	257	257	260	259	263	263	262	260	261	265	266	265	267	268	272	272	271	272	272	276	275	
	No. 3	242	240	245	250	250	250	252	258	257	258	263	261	266	262	262	264	269	270	274	279	277	283	278	
	No. 4	213	213	218	220	217	219	217	223	220	221	223	225	227	221	227	225	225	221	225	227	224	227	223	
	No. 5	228	226	244	249	240	240	247	249	250	253	260	255	257	256	258	260	270	274	271	278	275	285	282	
	No. 6	195	192	197	198	196	196	203	210	227	223	224	221	224	221	224	223	231	234	243	243	245	250	250	
	No. 7	262	258	274	276	266	272	278	280	286	286	285	286	289	286	287	290	295	291	299	307	303	310	310	
	No. 8	247	243	260	256	248	255	257	260	258	259	258	261	264	265	268	265	274	275	272	278	282	297	297	
	No. 9	189	190	198	198	200	200	207	217	233	228	233	231	232	235	236	240	242	243	247	248	247	255	255	
	No. 10	215	211	225	229	224	227	231	233	235	236	238	235	237	235	236	238	246	246	245	249	248	255	252	
	k 平均	230.5	229.3	240.0	241.8	237.6	239.6	244.6	247.8	251.3	251.2	253.2	252.4	255.1	252.9	255.5	256.0	261.3	261.4	263.8	267.9	267.4	274.4	272.8	
	m 野菜交付量 (g)	キャベツ 10 q							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

Table 6-2

第 A IV 群

a 実験月日 (1962)		1/1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
b 実験日数		89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
c 飼育温度 (°C)		22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22
d ラット体重 (g)	No. 1	302	305	308	308	306	307	306	310	311	310	307	308	307	314	310	319	322	323	324	326	330	333	333
	No. 2	277	279	280	273	274	278	279	280	285	284	282	283	286	293	287	297	300	297	298	300	299	304	300
	No. 3	271	283	292	287	282	291	290	290	294	286	285	286	286	290	287	293	297	296	295	301	298	296	303
	No. 4	227	227	232	230	240	236	233	240	240	232	229	232	232	232	230	241	244	240	237	239	240	242	243
	No. 5	283	288	288	284	285	285	285	284	290	285	286	285	280	288	284	288	297	292	291	294	282	291	293
	No. 6	248	251	250	253	255	253	257	254	257	253	253	257	253	259	257	266	270	267	268	276	275	277	275
	No. 7	304	313	310	312	316	312	316	313	320	315	315	313	313	316	311	317	318	318	316	321	321	329	324
	No. 8	302	312	306	310	316	315	319	321	324	318	312	311	315	317	311	320	319	318	320	320	318	318	320
	No. 9	250	253	253	254	258	258	261	256	266	263	263	268	267	274	269	270	270	272	273	275	270	267	269
	No. 10	252	256	253	253	255	255	254	256	257	256	251	254	254	260	257	264	267	262	264	264	264	266	266
	e 平均	271.6	276.7	277.2	276.4	278.7	279.0	280.0	280.4	284.4	280.2	278.3	279.7	279.3	284.3	280.3	287.5	290.4	288.5	288.6	291.6	289.7	292.3	292.6
f 野菜交付量 (g)							キャベツ 10	n						キャベツ 10	o						キャベツ 10	p		

g 実験月日 (1962)		24	25	26	27	28	29	30	31	1/2	2	3	4	5	6	7	8	9	10	11	12	13	14	15
h 実験日数		112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
i 飼育温度 (°C)		22	21	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22
j ラット体重 (g)	No. 1	323	333	329	330	330	332	333	333	328	330	330	334	335	335	328	332	330	333	331	335	334	330	335
	No. 2	296	300	300	300	301	307	307	311	304	302	304	306	310	306	302	304	303	303	302	309	308	308	306
	No. 3	299	303	304	299	301	304	306	306	300	302	303	306	308	306	304	300	301	298	300	308	307	306	304
	No. 4	241	245	245	244	239	249	253	254	255	255	258	260	259	259	258	255	255	254	257	260	257	262	262
	No. 5	289	293	293	290	291	295	295	296	295	292	294	297	292	290	286	298	292	288	288	290	294	290	290
	No. 6	268	275	276	276	278	282	285	281	281	281	278	287	285	285	286	285	285	284	287	287	293	293	295
	No. 7	321	326	324	323	320	328	326	328	327	326	320	326	332	330	324	324	325	323	325	332	331	329	332
	No. 8	316	322	317	317	318	320	320	320	316	317	320	319	320	321	313	315	318	314	316	318	329	320	323
	No. 9	271	275	277	273	276	277	282	285	282	283	280	286	284	285	277	280	282	283	287	292	293	294	295
	No. 10	259	261	261	260	261	266	262	262	262	259	264	269	268	267	263	260	261	264	263	273	273	271	268
	k 平均	288.3	293.3	292.6	291.2	291.5	296.0	296.9	297.6	295.0	294.7	295.1	298.8	299.3	298.4	294.1	295.3	295.2	294.4	295.6	300.4	301.9	300.3	301.0
m 野菜交付量 (g)					キャベツ 10	q						キャベツ 10	r						キャベツ 10	s				

Table 6-2

a 実験月日 (1962)		16/III	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
b 実験日数		135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
c 飼育温度 (°C)		22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
d ラット体重 (g)	No. 1	335	333	317	336	334	330	334	343	344	345	345	345	346	349	349	345	344	359	354	354	349	352	353
	No. 2	305	303	310	312	309	307	310	317	320	323	326	324	320	320	326	321	319	336	330	327	326	325	330
	No. 3	299	296	306	309	306	305	303	311	310	309	319	317	313	312	312	312	308	331	319	322	318	319	319
	No. 4	263	257	278	272	269	270	265	277	277	278	280	283	280	283	285	284	286	285	284	289	284	277	280
	No. 5	290	290	294	295	295	294	294	300	302	304	309	306	304	304	306	306	306	313	310	313	310	309	311
	No. 6	295	292	295	295	301	304	301	310	306	302	303	307	304	312	312	312	310	315	317	315	308	311	313
	No. 7	333	331	332	334	336	340	338	344	345	347	346	348	348	352	350	350	348	362	364	361	355	356	356
	No. 8	320	320	322	325	323	327	327	340	332	328	337	337	335	335	330	335	332	346	346	347	335	339	340
	No. 9	293	294	297	300	303	303	305	310	311	313	312	316	316	317	320	316	312	303	314	315	312	312	312
	No. 10	267	265	272	282	275	275	277	279	279	279	286	280	279	280	279	279	278	292	288	286	283	283	282
e 平均		300.0	298.1	301.5	306.0	305.1	305.5	305.4	313.1	312.6	312.8	316.3	316.3	314.5	316.5	316.9	316.0	314.3	324.2	322.6	322.9	318.0	318.3	139.6
f 野菜交付量 (g)			キャベツ 10 n							キャベツ 10 o							キャベツ 10 p							キャベツ 10 q
g 実験月日 (1962)		11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
h 実験日数		158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
i 飼育温度 (°C)		22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
j ラット体重 (g)	No. 1	352	356	352	352	351	351	352	354	362	361	362	366	365	366	365	368	365	365	363	361	365	360	365
	No. 2	331	341	337	335	331	333	330	336	344	341	351	352	346	346	348	356	347	346	355	350	352	348	354
	No. 3	317	319	319	315	313	316	313	318	328	325	327	330	326	325	322	326	329	334	328	329	332	326	336
	No. 4	280	286	284	283	284	286	283	288	289	300	295	296	297	300	298	300	303	300	300	298	294	297	303
	No. 5	314	320	313	317	313	313	312	314	323	328	330	320	322	324	324	327	327	329	329	327	330	327	334
	No. 6	313	320	319	315	312	319	313	322	324	327	323	330	324	330	324	333	332	328	331	326	330	332	332
	No. 7	355	363	360	358	357	358	353	362	368	367	364	368	365	364	364	374	369	366	366	363	367	363	371
	No. 8	345	348	345	345	342	342	342	345	355	350	347	355	351	350	349	357	350	350	345	347	346	344	354
	No. 9	317	318	320	320	322	319	320	322	319	320	324	326	325	328	324	331	332	330	327	323	330	327	330
	No. 10	285	290	282	284	283	281	282	284	292	287	289	289	290	289	286	297	291	289	289	286	287	288	299
k 平均		320.9	326.1	323.1	322.4	320.8	321.8	320.0	324.5	330.4	330.6	331.2	334.1	331.1	332.2	330.4	336.9	334.5	333.4	333.3	331.0	333.3	331.2	337.8
m 野菜交付量 (g)								キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

A 第IV群

Table 6-2

A	第IV群	a 実験月日 (1962)	16/II	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
		b 実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
		c 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
		d ラット体重 (g) No. 1	335	333	317	336	334	330	334	343	344	345	345	345	346	349	349	345	344	359	354	354	349	352	353
		No. 2	305	303	310	312	309	307	310	317	320	323	326	324	320	320	326	321	319	336	330	327	326	325	330
		No. 3	299	296	306	309	306	305	303	311	310	309	319	317	313	312	312	312	308	331	319	322	318	319	319
		No. 4	263	257	278	272	269	270	265	277	277	278	280	283	280	283	285	284	286	285	284	289	284	277	280
		No. 5	290	290	294	295	295	294	294	300	302	304	309	306	304	304	306	306	306	313	310	313	310	309	311
		No. 6	295	292	295	295	301	304	301	310	306	302	303	307	304	312	312	312	310	315	317	315	308	311	313
		No. 7	333	331	332	334	336	340	338	344	345	347	346	348	348	352	350	350	348	362	364	361	355	356	356
		No. 8	320	320	322	325	323	327	327	340	332	328	337	337	335	335	330	335	332	346	346	347	335	339	340
		No. 9	293	294	297	300	303	303	305	310	311	313	312	316	316	317	320	316	312	303	314	315	312	312	312
		No. 10	267	265	272	282	275	275	277	279	279	279	286	280	279	280	279	279	278	292	288	286	283	283	282
		e 平均	300.0	298.1	301.5	306.0	305.1	305.5	305.4	313.1	312.6	312.8	316.3	316.3	314.5	316.5	316.9	316.0	314.3	324.2	322.6	322.9	318.0	318.3	139.6
		f 野菜交付量 (g)		キャベツ 10 n							キャベツ 10 o							キャベツ 10 p							キャベツ 10 q
	第III群	g 実験月日 (1962)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
		h 実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
		i 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
		j ラット体重 (g) No. 1	352	356	352	352	351	351	352	354	362	361	362	366	365	366	365	368	365	365	363	361	365	360	365
		No. 2	331	341	337	335	331	333	330	336	344	341	351	352	346	346	348	356	347	346	355	350	352	348	354
		No. 3	317	319	319	315	313	316	313	318	328	325	327	330	326	325	322	326	329	334	328	329	332	326	336
		No. 4	280	286	284	283	284	286	283	288	289	300	295	296	297	300	298	300	303	300	300	298	294	297	303
		No. 5	314	320	313	317	313	313	312	314	323	328	330	320	322	324	324	327	327	329	329	327	330	327	334
		No. 6	313	320	319	315	312	319	313	322	324	327	323	330	324	330	324	333	332	328	331	326	330	332	332
		No. 7	355	363	360	358	357	358	353	362	368	367	364	368	365	364	364	374	369	366	366	363	367	363	371
		No. 8	345	348	345	345	342	342	342	345	355	350	347	355	351	350	349	357	350	350	345	347	346	344	354
		No. 9	317	318	320	320	322	319	320	322	319	320	324	326	325	328	324	331	332	330	327	323	330	327	330
		No. 10	285	290	282	284	283	281	282	284	292	287	289	289	290	289	286	297	291	289	289	286	287	288	299
		k 平均	320.9	326.1	323.1	322.4	320.8	321.8	320.0	324.5	330.4	330.6	331.2	334.1	331.1	332.2	330.4	336.9	334.5	333.4	333.3	331.0	333.3	331.2	337.8
		m 野菜交付量 (g)							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

Table 7-1

a 実験月日 (1961)		30/IX	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
b 実験日数		1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
c 飼育温度 (°C)		22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	20	21	21
d ラット体重 (g)	No. 1	80	79	93	100	104	t 交付開始	100	101	105	106	108	111	115	116	121	122	127	130	136	136	138	137	141	134
	No. 2	75	75	82	87	86		91	91	97	98	100	104	107	112	117	119	119	124	126	127	128	130	134	136
	No. 3	76	77	84	92	93		97	99	103	107	110	111	113	118	123	121	128	130	133	133	134	137	141	113
	No. 4	72	73	83	89	90		95	93	106	99	103	106	108	112	117	117	118	125	127	127	128	128	132	138
	No. 5	71	70	80	82	89		94	94	102	104	107	108	110	112	117	118	122	123	125	127	132	134	136	138
	No. 6	70	72	81	85	90		94	93	102	104	106	109	111	117	120	120	122	123	127	127	129	128	133	131
	No. 7	74	72	81	84	93		96	102	109	107	112	116	116	119	123	122	124	124	126	131	133	134	136	138
	No. 8	68	66	76	82	87		92	93	98	100	107	107	109	110	112	113	116	119	121	124	125	126	130	130
	No. 9	64	67	76	80	86		91	91	97	98	101	102	101	104	106	108	109	110	114	115	115	115	121	122
	No. 10	72	70	77	79	79		88	90	96	95	100	100	104	107	111	111	115	117	121	122	124	125	135	125
	e 平均	72.2	72.1	81.3	86.0	89.7		93.8	94.7	101.5	101.8	105.4	107.4	109.4	112.7	116.7	117.1	120.0	122.5	125.6	126.9	128.6	129.4	133.9	133.5
f 野菜交付量 (g)									n	キャベツ 10						o	キャベツ 10						p	キャベツ 10	

g 実験月日 (1961)	23	24	25	26	27	28	29	30	31	1/XI	2	3	4	5	6	7	8	9	10	11	12	13	14
h 実験日数	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
i 飼育温度 (°C)	21	21	21	22	23	23	23	23	23	23	22	13	23	23	21	22	22	22	22	22	22	21	21
j ラット体重 (g) No. 1	146	147	150	151	155	164	164	171	172	174	175	179	178	184	186	190	191	195	194	198	197	196	198
No. 2	136	133	135	135	138	142	142	147	140	144	145	151	152	152	152	160	161	165	162	162	165	168	170
No. 3	146	147	150	152	151	158	159	165	161	163	167	172	172	176	172	179	180	182	183	189	187	189	189
No. 4	139	139	141	143	143	152	154	159	156	157	158	163	166	170	170	176	177	176	182	187	189	188	190
No. 5	140	142	144	146	146	154	156	162	159	162	162	166	168	171	171	176	174	178	180	184	184	187	187
No. 6	135	137	139	142	142	145	150	158	154	154	157	160	154	167	170	170	170	174	171	174	175	178	177
No. 7	141	141	146	145	147	155	154	161	160	161	165	169	168	172	170	173	179	179	182	185	185	188	190
No. 8	131	130	130	130	133	137	140	145	142	150	149	153	155	156	160	162	165	168	169	176	177	181	180
No. 9	125	122	125	125	125	131	130	139	137	138	138	143	140	150	150	158	161	164	163	171	170	172	170
No. 10	128	127	130	132	133	137	138	137	140	143	142	140	141	135	140	143	141	145	146	150	151	151	154
k 平均	136.7	136.5	139.0	140.1	141.3	147.5	150.7	154.4	152.1	154.6	155.8	159.6	159.4	163.3	164.7	168.1	169.9	172.6	173.2	177.6	178.0	179.8	180.5
m 野菜交付量 (g)						キャベツ 10 q							キャベツ 10 r							キャベツ 10 s			

Table 7-1

a 実験月日 (1961)		15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
b 実験日数		42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
c 飼育温度 (°C)		21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
d ラット体重 (g)	No. 1	198	198	198	208	212	211	210	203	215	213	224	223	221	236	231	228	225	235	234	232	237	235	240	240
	No. 2	172	174	178	188	193	190	194	195	202	198	204	205	204	207	212	212	214	218	217	217	220	222	226	223
	No. 3	194	195	195	203	209	207	204	209	208	209	218	217	216	216	217	215	214	221	221	229	222	225	228	225
	No. 4	195	197	195	207	213	217	216	221	225	220	230	230	231	236	238	234	232	241	248	247	251	252	256	253
	No. 5	194	195	195	205	208	211	214	215	219	217	228	227	224	228	236	233	230	239	241	242	241	241	245	241
	No. 6	180	180	180	186	193	189	193	197	199	205	212	210	206	215	217	216	213	221	222	224	225	225	227	226
	No. 7	188	194	193	202	210	206	208	217	216	217	228	229	228	234	238	236	235	243	254	249	254	257	258	258
	No. 8	184	188	186	193	200	200	202	205	204	202	217	212	218	216	220	221	212	221	223	228	229	227	227	220
	No. 9	175	177	178	183	188	189	188	191	190	196	199	200	208	204	205	204	198	205	206	210	212	211	214	213
	No. 10	155	157	155	156	160	160	160	160	163	163	164	167	166	176	187	194	192	208	206	205	211	206	215	215
e 平均		183.5	185.5	185.3	193.1	198.6	198.0	198.9	201.3	204.1	204.0	212.4	212.0	212.2	216.8	220.1	219.3	216.5	225.2	227.2	228.3	230.2	230.1	233.6	231.4
f 野菜交付量 (g)					キャベツ 10 n							キャベツ 10 o							キャベツ 10 p						
g 実験月日 (1961)		9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
h 実験日数		66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	
i 飼育温度 (°C)		22	22	22	22	22	22	22	23	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23	
j ラット体重 (g)	No. 1	245	243	255	266	255	262	264	265	269	275	273	270	274	275	275	272	281	275	275	283	288	294	282	
	No. 2	231	228	232	234	237	234	240	239	243	240	248	240	249	251	249	252	251	251	249	257	257	256	257	
	No. 3	230	226	243	246	236	241	247	249	249	253	253	247	250	252	250	250	264	259	261	268	269	277	271	
	No. 4	263	265	271	281	270	280	284	285	297	295	300	292	299	298	298	300	298	307	306	310	310	314	313	
	No. 5	249	247	263	264	257	258	263	261	266	273	272	265	271	269	273	272	280	278	282	283	289	290	288	
	No. 6	236	238	245	245	240	244	249	251	258	255	257	259	269	264	256	266	273	267	268	275	272	279	281	
	No. 7	265	264	275	279	273	276	282	281	287	285	293	283	291	295	292	290	292	292	287	296	295	305	303	
	No. 8	234	227	243	253	250	250	250	251	257	248	253	255	263	258	259	261	265	269	269	277	277	285	283	
	No. 9	215	214	233	228	228	228	231	233	235	238	242	240	243	244	241	239	245	245	245	659	249	251	249	
	No. 10	222	220	235	241	234	239	240	243	247	249	249	247	252	254	252	250	257	261	260	263	261	277	275	
k 平均		239.0	237.2	249.5	253.7	248.0	251.2	255.0	255.8	260.8	261.1	264.0	259.8	265.9	266.0	264.6	265.2	270.6	270.4	270.2	277.1	276.7	282.8	280.2	
m 野菜交付量 (g)		キャベツ 10 q							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

Table 7-2

第V群 A	a 実験月日 (1962)	1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	b 実験日数	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
	c 飼育温度 (°C)	22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22
	d ラット体重 (g) No. 1	294	300	297	303	302	306	309	312	310	306	301	304	308	313	306	309	314	310	312	314	317	313	315
	No. 2	256	261	260	261	262	262	267	263	265	266	263	261	262	268	262	265	268	266	274	273	278	273	277
	No. 3	272	277	278	280	277	281	278	280	282	285	278	277	278	295	289	289	296	290	292	298	283	296	303
	No. 4	318	315	313	319	314	322	321	319	321	317	315	318	319	334	323	331	334	330	333	335	333	332	336
	No. 5	295	293	289	292	295	300	301	300	306	300	296	299	296	304	297	303	305	302	303	306	306	300	302
	No. 6	281	286	288	282	285	287	293	289	291	293	290	300	297	304	296	300	300	296	298	298	301	306	302
	No. 7	299	308	313	308	306	312	314	312	310	313	307	308	305	312	309	304	315	313	313	322	322	316	324
	No. 8	281	283	288	286	296	289	293	293	297	294	286	300	298	300	287	300	304	300	297	301	298	299	304
	No. 9	246	249	253	250	256	256	256	260	262	262	264	264	270	272	267	271	280	275	278	278	271	273	277
	No. 10	277	284	284	285	283	288	290	287	291	287	289	287	289	291	284	290	291	295	293	296	296	294	299
	e 平均	281.9	285.6	286.3	286.6	287.6	270.3	292.2	291.5	293.5	292.3	288.1	291.8	292.2	299.1	297.0	296.2	300.7	297.7	299.3	302.1	301.1	300.2	303.9
	p 野菜交付量 (g)						キャベツ 10 n							キャベツ 10 o							キャベツ 10 p			
	q 実験月日 (1962)	24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	h 実験日数	112	113	114	115	116	117	118	119	20	121	122	123	124	125	126	127	128	129	130	131	132	133	134
	i 飼育温度 (°C)	22	21	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22
	j ラット体重 (g) No. 1	312	313	310	310	307	310	312	315	317	317	320	324	321	321	320	319	321	322	323	323	324	323	328
	No. 2	275	271	271	276	276	278	279	279	277	277	282	287	282	284	286	289	289	291	293	294	295	294	295
	No. 3	294	295	296	290	291	295	295	294	295	295	294	298	297	296	295	294	296	297	297	299	299	300	299
	No. 4	331	332	331	332	328	336	337	336	330	331	334	337	333	333	330	330	331	333	325	310	337	339	336
	No. 5	300	302	301	302	302	307	311	309	309	307	308	310	308	308	308	307	310	312	312	312	313	313	312
	No. 6	300	305	305	306	303	302	304	311	310	302	308	310	306	312	311	307	307	310	315	315	322	314	321
	No. 7	318	319	317	318	315	314	315	314	314	318	319	328	323	330	324	323	324	327	331	330	337	330	337
	No. 8	302	303	306	306	307	307	308	310	311	315	319	326	319	318	327	322	327	331	331	335	335	332	335
	No. 9	298	278	275	277	273	278	279	282	282	280	277	281	277	278	273	271	277	277	279	282	285	283	280
	No. 10	296	296	298	298	300	299	301	301	303	302	303	307	307	307	306	304	310	309	313	313	317	316	315
	k 平均	300.6	301.4	301.0	301.5	300.7	302.7	306.1	305.1	304.8	301.4	306.4	310.8	307.3	308.7	308.1	306.6	309.2	310.9	311.9	314.3	316.4	314.5	315.8
	m 野菜交付量 (g)				キャベツ 10 q							キャベツ 10 r							キャベツ 10 s					

Table 7-2

第V期	a 実験月日 (1962)	16/III	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
	b 実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
	c 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
	d ラット体重 (g) No. 1	320	322	326	324	336	329	330	332	332	334	334	336	338	341	343	340	340	351	346	349	345	347	345
	No. 2	296	291	296	295	300	299	302	299	300	303	298	302	306	306	308	305	305	310	307	309	308	308	307
	No. 3	296	295	298	296	298	297	298	299	301	302	306	304	304	305	305	302	304	321	311	311	310	310	314
	No. 4	339	337	338	337	342	341	342	342	312	342	347	347	346	352	352	346	352	310	358	356	351	353	356
	No. 5	314	309	310	312	314	319	315	322	320	320	323	323	318	325	326	324	324	332	333	333	327	329	326
	No. 6	316	316	328	316	317	318	320	323	327	330	329	330	330	335	335	328	334	343	336	335	335	331	337
	No. 7	335	332	336	331	340	334	335	341	340	338	338	348	342	346	350	346	348	355	357	353	317	353	353
	No. 8	333	330	332	325	334	335	332	337	336	338	342	347	346	342	343	342	346	353	349	351	349	319	345
	No. 9	284	276	286	278	283	282	282	281	285	288	286	290	285	288	288	277	280	287	291	294	286	295	293
	No. 10	317	315	315	317	319	320	321	324	328	334	330	332	334	335	333	333	336	349	341	341	337	312	343
	e 平均	315.0	312.3	316.5	313.1	318.3	317.4	317.7	320.0	321.0	323.9	323.3	325.9	324.9	327.5	328.3	324.3	326.9	334.1	332.9	333.5	329.5	332.0	331.9
	f 野菜交付量 (g)		キャベツ 10 n							キャベツ 10 o							キャベツ 10 p							キャベツ 10 q
A群 (2)	g 実験月日 (1962)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
	h 実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
	i 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
	j ラット体重 (g) No. 1	349	353	350	350	350	350	350	352	353	352	354	358	354	355	352	354	354	355	354	353	352	351	358
	No. 2	310	309	313	315	309	311	310	312	318	314	314	320	316	312	313	320	314	312	314	314	315	314	317
	No. 3	312	313	313	314	314	314	313	318	326	324	319	330	324	326	324	329	324	325	327	324	327	328	336
	No. 4	356	356	358	357	358	357	360	358	360	364	365	358	362	362	360	364	365	365	366	362	364	363	366
	No. 5	333	332	331	331	332	331	334	332	338	342	342	340	336	337	338	336	337	337	337	338	336	338	342
	No. 6	340	349	345	348	345	344	344	342	351	357	353	353	347	348	348	357	350	350	352	354	353	349	360
	No. 7	356	356	356	362	356	362	361	358	365	367	362	369	362	360	363	362	358	364	364	364	364	362	369
	No. 8	351	355	356	351	353	351	357	356	362	365	359	360	359	363	358	365	368	372	371	370	373	371	376
	No. 9	292	297	298	297	298	295	295	292	298	302	309	310	304	304	298	305	305	303	302	302	302	305	305
	No. 10	343	353	349	346	346	343	345	344	350	347	345	347	346	345	346	352	348	347	346	346	347	348	356
	k 平均	334.2	337.3	336.9	337.1	336.1	335.8	336.9	336.4	342.1	343.4	342.2	341.5	341.0	341.2	340.0	344.4	342.3	343.0	343.3	342.7	343.3	342.9	348.5
	m 野菜交付量 (g)							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

Table 8-1

第VI群	a 実験月日 (1962)		30/IV	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
	b 実験日数		1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
	c 飼育温度 (°C)		22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21
	d ラット体重 (g)	No. 1	88	87	98	104	114	t 交付開始	119	116	122	123	124	131	131	134	137	131	139	143	143	143	142	143	149	149
		No. 2	79	77	89	89	102		107	108	119	118	123	120	121	127	129	128	131	132	136	137	138	141	144	118
		No. 3	79	82	90	93	103		111	110	118	118	117	121	126	127	132	131	135	137	139	143	142	145	151	151
		No. 4	77	76	85	91	94		98	99	106	109	111	109	116	116	117	120	121	123	122	118	115	126	121	122
		No. 5	69	71	80	87	86		92	95	99	100	104	104	109	111	115	114	115	118	122	122	125	120	128	130
		No. 6	71	70	81	85	87		95	95	103	106	111	108	110	115	118	120	121	127	130	130	132	134	141	137
		No. 7	67	69	78	84	90		98	99	106	108	110	109	113	116	121	120	122	128	130	131	132	134	138	140
		No. 8	68	64	75	82	86		95	96	104	106	109	113	115	117	123	122	127	133	135	137	139	137	141	140
		No. 9	69	69	76	81	83		88	93	96	100	104	107	103	112	116	111	119	126	127	127	129	130	135	136
		No. 10	63	62	71	79	84		84	90	98	96	99	99	97	103	104	106	108	109	113	114	117	117	121	121
	e 平均		73.0	72.7	82.3	87.5	92.9		98.7	99.8	107.1	108.4	111.2	112.1	114.1	117.8	121.2	120.3	123.8	127.6	129.7	130.2	131.1	132.7	136.1	137.4
	f 野菜交付量 (g)										キャベツ 10	n						キャベツ 10	o						キャベツ 10	p
群	g 実験月日 (1962)		23	24	25	26	27	28	29	30	31	1/XI	2	3	4	5	6	7	8	9	10	11	12	13	14	
	h 実験日数		19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	
	i 飼育温度 (°C)		21	21	21	22	23	23	23	23	23	23	22	23	23	23	21	22	22	22	22	22	22	21	21	
	j ラット体重 (g)	No. 1	151	150	155	154	155	158	163	170	166	171	170	178	175	178	179	183	188	189	188	193	191	197	199	
		No. 2	146	145	148	152	155	157	169	172	167	169	170	173	169	180	176	180	185	186	189	196	193	201	199	
		No. 3	154	150	155	157	158	162	170	173	170	173	174	176	178	183	180	184	189	189	190	190	189	194	195	
		No. 4	128	128	123	135	132	137	138	134	130	135	137	142	143	150	150	153	157	162	163	166	168	171	193	
		No. 5	131	130	135	136	136	142	145	154	150	153	155	160	159	159	167	170	173	174	176	181	180	190	190	
		No. 6	140	137	141	142	142	149	150	153	153	154	155	161	161	168	161	168	169	171	172	181	180	181	180	
		No. 7	140	139	143	143	143	148	151	155	152	154	156	160	156	167	162	168	170	171	172	180	179	182	185	
		No. 8	145	147	150	152	154	164	164	172	169	173	174	179	179	180	182	188	190	193	193	197	197	199	201	
		No. 9	139	138	140	142	143	150	154	160	158	159	160	166	166	174	172	178	177	180	180	183	182	190	189	
		No. 10	124	123	126	128	130	138	138	146	142	146	147	151	153	156	158	159	163	164	165	169	167	170	172	
	k 平均		139.8	138.7	141.6	144.1	144.8	150.5	154.2	158.9	155.7	158.7	159.8	164.6	163.9	169.5	168.7	173.1	176.1	177.9	178.8	183.6	182.6	187.5	188.3	
	m 野菜交付量 (g)							キャベツ 10	q						キャベツ 10	r						キャベツ 10	s			

A

Table 8-1

第VI期	a 実験月日 (1962)	15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
	b 実験日数	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
	c 飼育温度 (°C)	21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
	d ラット体重 (g) No. 1	200	201	201	208	216	214	214	222	221	226	233	235	234	242	245	244	241	250	253	253	254	260	264	262
	No. 2	202	201	202	211	217	218	215	220	221	224	231	233	228	234	237	233	232	240	241	243	242	246	248	250
	No. 3	198	197	196	206	213	214	214	218	220	222	230	230	228	229	235	234	230	242	243	242	246	247	249	249
	No. 4	175	173	177	184	190	187	192	193	225	215	205	205	200	206	208	207	202	213	216	217	219	218	220	220
	No. 5	192	194	195	204	212	207	206	205	201	224	235	232	228	236	233	244	232	245	256	245	253	256	259	254
	No. 6	183	183	182	187	194	195	196	203	206	205	212	212	207	211	215	216	209	219	225	225	227	225	227	231
	No. 7	189	191	183	200	199	195	198	202	225	205	214	210	214	214	219	218	215	220	224	229	233	230	231	231
	No. 8	204	206	208	216	223	221	221	226	218	224	237	238	233	237	243	238	238	249	253	254	258	258	262	259
	No. 9	190	194	193	201	212	212	216	215	196	220	228	234	230	231	238	234	234	238	244	240	244	245	244	246
	No. 10	176	179	177	185	190	192	190	194	196	199	205	210	212	212	215	211	209	220	220	223	221	227	226	227
	e 平均	190.9	191.9	191.4	200.2	206.6	205.5	206.2	209.8	212.9	214.4	223.0	223.9	222.4	225.2	228.8	227.9	224.2	233.6	237.0	237.1	239.7	242.2	243.0	242.9
	f 野菜交付量 (g)				キャベツ 10 n							キャベツ 10 o							キャベツ 10 p						
第VII期	g 実験月日 (1962)	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
	h 実験日数	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	
	i 飼育温度 (°C)	22	22	22	22	22	22	22	23	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23	
	j ラット体重 (g) No. 1	269	271	280	284	285	285	288	291	290	294	298	291	296	294	290	290	294	292	291	296	290	289	289	
	No. 2	257	255	265	275	269	272	278	277	282	279	283	278	286	283	280	288	290	288	291	295	280	299	290	
	No. 3	256	254	266	273	264	272	278	279	278	276	285	282	289	282	285	282	295	295	294	300	295	305	299	
	No. 4	226	222	237	240	238	244	249	249	247	243	249	248	251	248	251	252	251	252	253	257	255	258	253	
	No. 5	261	260	270	279	276	277	282	285	289	287	291	290	295	293	291	290	293	292	296	299	294	303	302	
	No. 6	243	237	253	254	244	251	259	257	267	268	273	266	271	273	278	278	283	284	272	291	285	297	298	
	No. 7	237	235	253	255	245	250	255	254	263	259	264	267	272	268	270	272	274	278	276	282	278	299	296	
	No. 8	271	269	284	285	277	286	289	289	297	295	299	301	304	301	297	297	301	302	295	304	300	320	327	
	No. 9	250	248	253	257	256	257	260	264	265	264	267	264	263	268	266	270	271	272	271	275	274	280	277	
	No. 10	239	235	249	258	246	253	260	260	267	268	268	266	276	274	275	275	285	293	290	294	290	301	300	
	k 平均	250.9	248.6	261.0	266.0	260.0	264.7	269.7	270.5	274.5	275.4	277.7	275.3	280.3	278.4	278.3	279.4	283.7	284.8	292.9	289.3	284.8	295.1	293.1	
	m 野菜交付量 (g)	キャベツ 10 q							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

Table 8-2

a 実験月日 (1962)	1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
b 実験日数	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
c 飼育温度 (°C)	22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22
第VI群 A d ラット体重 (g) No. 1	290	294	293	286	289	294	296	293	299	298	287	295	291	292	291	300	299	298	299	302	303	305	311
No. 2	292	295	299	292	296	297	298	296	301	295	298	303	298	308	303	303	305	306	302	304	307	305	310
No. 3	301	303	307	302	301	304	306	305	314	310	303	306	308	317	307	314	315	312	311	314	314	311	315
No. 4	258	257	259	257	257	265	266	259	273	272	266	271	277	284	277	275	283	277	283	288	288	279	289
No. 5	302	300	301	300	302	297	300	289	306	303	300	303	301	309	303	308	315	311	307	312	308	309	315
No. 6	297	300	296	300	301	303	305	306	308	314	310	308	303	314	307	312	318	320	322	324	328	323	334
No. 7	300	314	283	305	305	289	304	311	306	306	298	304	302	313	303	312	313	312	315	315	315	321	319
No. 8	336	324	330	344	341	335	338	349	344	340	337	345	310	355	343	350	353	352	346	354	352	355	359
No. 9	286	280	278	280	281	279	283	277	284	283	280	286	284	288	283	290	287	290	292	297	301	298	303
No. 10	304	309	300	307	310	313	317	311	316	319	312	320	319	330	313	316	331	331	333	338	336	339	314
e 平均	296.6	297.6	294.6	297.3	298.3	297.6	301.5	299.6	305.1	304.0	299.1	304.1	302.3	311.0	303.0	308.0	311.9	310.9	311.0	314.8	315.2	314.5	319.9
f 野菜交付量 (g)						キャベツ 10 n							キャベツ 10 o							キャベツ 10 p			
g 実験月日 (1962)	24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15
h 実験日数	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	11	130	131	132	133	134
i 飼育温度 (°C)	22	12	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22
第VI群 j ラット体重 (g) No. 1	308	309	309	310	306	313	310	312	310	311	317	318	313	316	314	314	316	315	321	322	323	324	326
No. 2	301	302	305	303	302	303	308	304	304	300	304	308	305	306	303	306	307	308	311	310	311	310	311
No. 3	313	318	316	313	317	313	315	316	316	316	318	327	321	322	319	321	322	320	324	324	333	327	331
No. 4	288	286	287	280	291	292	295	299	298	298	298	303	296	301	301	302	298	301	304	307	308	307	299
No. 5	306	308	308	306	307	306	305	306	307	307	308	309	307	311	309	308	310	310	312	310	314	314	315
No. 6	321	323	320	322	319	330	330	329	332	329	331	311	336	335	330	332	335	310	311	313	312	313	314
No. 7	317	317	322	327	[illegible]	326	325	324	321	333	329	332	326	333	328	335	332	332	335	337	318	338	310
No. 8	354	354	355	355	[illegible]	353	355	357	363	363	361	370	361	355	362	367	368	363	368	366	370	368	371
No. 9	298	298	298	297	298	301	304	300	300	302	303	301	301	301	300	300	302	302	303	307	307	304	305
No. 10	337	338	338	337	339	337	335	338	341	338	337	313	311	312	312	312	312	317	319	351	352	319	353
k 平均	314.3	315.3	305.8	315.0	316.1	317.4	318.2	318.5	319.5	319.4	320.9	325.6	320.7	322.8	320.8	322.7	323.2	323.8	326.8	327.7	331.3	328.4	329.5
m 野菜交付量 (g)				キャベツ 10 q							キャベツ 10 r							キャベツ 10 s					

Table 8-2

第VI群	a 実験月日 (1962)	16/III	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
	b 実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
	c 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
	d ラット体重 (g) No. 1	323	320	324	325	328	330	330	334	334	336	341	340	340	345	347	345	347	357	353	353	347	352	351
	No. 2	311	305	312	312	315	314	314	320	320	316	325	326	325	334	333	326	332	339	337	337	328	332	336
	No. 3	335	327	334	331	333	336	337	342	340	332	342	343	341	344	348	342	346	357	351	353	347	350	346
	No. 4	306	303	308	309	318	315	317	320	317	316	322	326	324	327	330	327	330	333	331	334	335	335	338
	No. 5	313	310	314	313	316	315	316	323	320	318	322	324	323	325	329	326	332	338	333	335	333	331	330
	No. 6	346	342	346	343	356	354	353	357	356	356	362	361	366	364	367	363	365	364	372	373	367	371	378
	No. 7	338	335	341	334	343	347	340	345	344	344	345	345	345	350	350	351	350	362	368	363	359	356	357
	No. 8	368	364	370	365	373	372	373	375	373	374	377	377	381	386	380	380	380	391	384	388	383	392	384
	No. 9	304	300	311	308	315	311	308	312	310	304	316	323	322	327	319	318	325	325	313	335	319	329	322
	No. 10	351	348	350	354	359	355	356	353	351	346	354	356	356	356	358	355	347	363	359	356	353	357	351
	e 平均	329.5	325.4	331.0	329.4	335.6	334.9	334.4	338.1	336.5	334.2	340.6	342.1	342.3	345.8	346.1	343.3	345.4	352.9	350.1	352.7	347.1	350.5	349.3
	f 野菜交付量 (g)		キャベツ 10 n							キャベツ 10 o							キャベツ 10	p						キャベツ 10 q
A群	g 実験月日 (1962)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
	h 実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
	i 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
	j ラット体重 (g) No. 1	356	350	350	350	346	344	342	342	326	332	329	325	322	317	311	309	303	312	313	316	317	320	326
	No. 2	337	341	340	342	337	339	340	340	341	349	345	348	345	346	345	346	346	346	346	343	345	341	348
	No. 3	352	350	353	352	349	352	353	348	356	358	355	359	360	355	352	350	350	353	356	352	355	352	358
	No. 4	336	336	341	340	340	338	338	340	339	343	342	343	347	346	346	344	346	348	348	346	341	347	350
	No. 5	333	329	336	333	335	334	332	334	341	343	340	340	339	338	339	345	342	345	340	342	341	340	343
	No. 6	370	370	369	378	377	383	379	380	383	385	380	386	384	384	382	389	387	388	384	389	387	383	400
	No. 7	354	356	354	361	362	355	357	361	359	360	362	366	364	372	366	372	367	368	364	362	364	362	367
	No. 8	384	385	383	386	384	387	384	390	396	396	390	389	389	388	386	393	391	389	386	387	388	387	403
	No. 9	324	329	329	328	324	326	323	330	333	334	332	329	333	330	328	336	331	332	335	332	332	330	337
	No. 10	354	352	353	350	351	348	353	350	356	359	353	355	350	350	352	362	359	356	360	357	357	353	359
	k 平均	350.0	349.2	350.8	352.0	350.5	350.6	350.1	351.4	353.0	353.2	352.8	354.0	353.3	352.6	350.7	354.6	352.2	353.7	353.2	352.6	352.7	351.5	359.1
	m 野菜交付量 (g)							キャベツ 10 r							キャベツ 10 s							キャベツ 10 t		

Table 9-1

a 実験月日 (1962)		30/IX	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
b 実験日数		1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
c 飼育温度 (°C)		22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21
d ラット体重 (g)	No. 1	89	85	97	105	106	t 交付開始	103	101	112	112	114	113	117	121	125	124	123	128	132	133	132	132	137	136
	No. 2	85	81	93	96	103		106	103	113	117	121	121	122	124	131	130	130	134	136	139	139	139	141	141
	No. 3	80	79	87	93	96		92	96	104	106	108	110	110	115	121	127	120	123	123	125	125	126	129	126
	No. 4	74	73	83	91	92		94	94	105	108	113	114	117	120	125	127	128	127	128	130	132	129	136	134
	No. 5	71	69	80	87	91		94	95	100	102	109	114	118	118	126	127	128	134	131	128	122	124	119	122
	No. 6	72	70	79	85	91		93	94	101	104	108	112	117	116	120	120	125	125	132	130	128	130	133	134
	No. 7	72	69	79	84	90		91	91	97	101	108	105	107	112	114	119	121	125	126	126	128	128	133	132
	No. 8	80	78	85	83	94		98	101	96	105	110	116	118	114	120	120	122	122	126	127	127	129	130	130
	No. 9	68	69	77	81	88		81	90	95	100	104	100	103	106	110	109	112	111	118	119	120	121	126	128
	No. 10	67	65	76	79	83		87	90	96	101	105	106	107	104	115	114	120	114	117	116	116	117	124	130
	e 平均	75.8	73.8	83.6	88.4	93.4		93.9	95.5	101.9	105.6	110.0	111.1	113.6	115.0	120.7	121.7	122.9	124.3	126.9	127.3	126.9	127.5	130.8	131.3
f 野菜交付量 (g)										†ャベツ 10 n							†ャベツ 10 o							†ャベツ 10 p	

g 実験月日 (1962)	23	24	25	26	27	28	29	30	31	1/XI	2	3	4	5	6	7	8	9	10	11	12	13	14
h 実験日数	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
i 飼育温度 (°C)	21	21	21	22	23	23	23	23	23	23	22	22	22	22	22	22	22	22	22	22	22	22	22
j ラット体重 (g) No. 1	139	141	145	146	146	154	155	159	160	165	165	168	169	172	175	178	182	182	180	186	184	185	187
No. 2	142	140	146	145	145	153	158	159	157	157	160	163	165	170	170	172	175	179	179	184	185	186	184
No. 3	129	130	133	132	133	139	143	152	146	148	150	154	152	155	155	161	163	160	160	169	169	173	172
No. 4	139	138	138	139	139	145	147	154	152	155	155	159	157	164	163	167	172	174	174	179	178	178	181
No. 5	129	128	128	129	129	134	138	143	143	144	147	150	154	155	156	156	159	158	158	162	161	161	166
No. 6	139	140	142	145	145	152	152	161	159	160	155	166	161	167	166	170	173	174	173	175	176	177	178
No. 7	132	135	137	137	139	143	145	145	153	148	151	154	156	157	154	158	163	163	161	170	169	170	170
No. 8	133	133	137	137	137	141	142	149	146	146	148	152	152	155	153	158	163	161	163	170	170	169	170
No. 9	129	130	131	131	133	140	144	155	150	152	152	156	152	166	151	165	170	171	172	176	176	178	180
No. 10	125	125	128	129	129	138	138	146	148	142	150	151	148	151	152	156	159	158	158	160	161	162	163
k 平均	133.0	134.0	136.5	137.0	137.5	143.8	146.2	152.3	151.4	152.0	153.3	157.3	156.6	161.5	159.5	161.1	167.9	168.3	168.8	173.1	172.9	174.4	175.1
m 野菜交付量 (g)						†ャベツ 10 q							†ャベツ 10 r							†ャベツ 10 s			

第VII群 A

Table 9-1

実験月日 (1962)	15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
実験日数	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
飼育温度 (°C)	21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	22
ラット体重 (g) No. 1	190	193	190	201	210	205	209	211	215	214	228	226	223	229	233	230	225	234	241	243	211	242	241	241
No. 2	192	193	190	201	214	208	209	211	213	213	228	226	222	226	229	226	225	237	236	237	238	242	241	243
No. 3	175	178	178	184	196	192	195	500	201	201	213	215	212	216	222	218	215	222	227	230	237	233	236	233
No. 4	185	187	185	196	200	200	205	210	212	217	227	225	221	224	229	227	225	235	237	240	211	241	215	242
No. 5	170	173	175	183	189	189	194	194	197	198	208	210	208	212	216	217	216	222	223	225	228	230	229	231
No. 6	182	183	183	190	200	194	196	211	215	213	216	219	212	222	225	222	227	225	231	229	231	233	230	237
No. 7	179	179	183	189	191	191	198	200	203	202	205	203	203	210	217	216	213	215	219	220	222	225	225	225
No. 8	174	178	178	183	195	190	193	205	303	203	212	215	209	215	220	219	219	228	229	233	234	238	240	237
No. 9	187	190	188	198	207	206	208	212	213	217	227	230	225	226	231	236	234	243	244	251	256	257	256	259
No. 10	168	169	171	176	178	180	185	186	191	194	204	205	201	210	212	214	211	215	218	218	222	218	225	221
平均	180.2	182.3	182.1	190.1	198.0	195.5	199.2	204.0	206.3	207.2	216.8	218.4	213.0	219.0	223.4	222.5	221.0	227.6	230.5	232.6	235.0	235.9	237.0	236.9
野菜交付量 (g)				ナッペ ツ 10							ナッペ ツ 10							ナッペ ツ 10						

実験月日 (1962)	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
実験日数	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88
飼育温度 (°C)	22	22	22	22	22	22	22	22	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23
ラット体重 (g) No. 1	249	249	225	259	251	257	264	259	265	265	269	264	267	268	268	270	275	274	271	285	283	299	295
No. 2	249	219	265	273	264	270	275	273	277	279	281	287	294	295	293	295	292	298	302	298	302	308	303
No. 3	241	237	248	256	256	254	257	257	257	263	265	266	264	267	269	271	273	276	278	281	277	283	277
No. 4	251	248	263	264	257	262	264	267	271	274	277	275	277	281	280	282	283	288	294	298	300	316	303
No. 5	231	235	244	248	243	243	245	246	254	255	262	255	261	262	259	260	261	258	263	275	270	276	273
No. 6	237	239	244	248	248	247	255	255	255	254	258	256	260	263	262	262	268	268	269	271	268	277	263
No. 7	228	230	231	238	235	238	243	239	240	245	245	245	252	251	253	254	261	257	258	261	260	262	258
No. 8	245	242	258	260	253	260	263	263	268	273	273	274	278	278	280	279	283	280	282	290	300	308	308
No. 9	261	260	270	247	262	271	275	273	285	277	277	284	286	288	285	288	287	292	294	304	301	312	306
No. 10	225	225	238	232	228	231	236	231	231	233	233	237	238	238	238	238	241	241	243	248	244	247	247
平均	241.7	241.4	251.6	255.2	250.2	253.3	257.7	256.3	253.3	261.8	265.9	264.3	267.7	269.1	268.7	269.9	272.4	273.2	274.4	281.1	280.8	288.8	283.3
野菜交付量 (g)	ナッペ ツ 10							ナッペ ツ 10							ナッペ ツ 10							ナッペ ツ 10	

Table 9-2

a 実験月日 (1962)		1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
b 実験日数		89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
c 飼育温度 (°C)		22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22
d ラット体重 (g)	No. 1	293	295	294	288	293	291	296	295	294	293	290	291	288	295	284	299	295	293	290	296	295	293	300
	No. 2	309	310	317	314	318	315	317	315	314	309	312	316	313	315	305	317	321	316	314	319	320	323	323
	No. 3	275	275	281	274	275	278	288	275	283	282	284	289	283	285	288	281	293	290	293	293	300	293	300
	No. 4	315	314	319	317	318	323	317	322	317	312	310	315	315	317	309	319	321	321	320	323	324	316	324
	No. 5	275	273	279	275	278	279	281	279	282	278	280	280	286	288	277	290	296	291	290	293	294	290	297
	No. 6	265	273	274	269	270	273	277	267	277	280	277	278	278	289	274	283	287	289	290	292	290	290	302
	No. 7	252	263	263	263	263	265	265	270	273	276	275	279	282	285	279	284	281	286	293	290	290	292	294
	No. 8	309	311	314	317	318	319	317	315	314	313	314	316	317	319	309	322	323	317	321	322	316	319	326
	No. 9	311	312	311	309	319	308	316	315	313	310	314	320	317	319	316	318	321	319	322	325	327	323	328
	No. 10	243	244	242	242	243	241	243	244	247	242	246	250	250	254	250	252	255	255	258	262	264	260	266
	e 平均	284.7	287.0	289.4	286.8	289.5	290.2	291.7	289.7	291.4	289.5	270.2	293.4	292.9	296.6	289.1	296.5	299.3	297.7	299.1	301.5	302.0	299.9	306.0
f 野菜交付量 (g)							キャベツ 10 n							キャベツ 10 o							キャベツ 10 p			
g 実験月日 (1962)		24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15
h 実験日数		112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
i 飼育温度 (°C)		22	21	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22
j ラット体重 (g)	No. 1	292	294	295	290	289	294	290	287	287	285	288	294	290	287	290	289	293	292	297	294	294	294	293
	No. 2	320	322	318	315	315	318	312	315	311	313	315	321	316	319	314	314	315	314	316	318	321	313	318
	No. 3	290	287	292	285	289	292	287	292	284	285	291	299	293	292	291	292	294	293	302	299	298	297	300
	No. 4	319	319	319	311	312	310	308	309	306	306	308	308	304	305	304	303	303	302	303	300	301	301	300
	No. 5	293	294	291	291	291	294	293	294	291	295	298	299	294	294	293	293	294	294	297	296	296	295	299
	No. 6	292	295	293	293	293	299	294	296	298	293	291	303	294	296	292	290	294	294	296	299	298	291	298
	No. 7	291	292	297	294	291	296	293	297	297	295	299	304	290	297	294	295	298	300	302	298	303	305	306
	No. 8	322	318	316	314	317	317	317	317	316	315	318	322	315	317	315	315	320	317	317	322	322	316	319
	No. 9	322	330	324	325	325	323	320	322	318	318	322	323	318	323	320	320	321	324	324	329	327	322	326
	No. 10	254	255	250	255	257	257	259	261	258	257	260	258	261	261	263	262	264	266	267	266	271	268	271
	k 平均	299.5	300.4	299.5	297.3	297.9	300.0	297.3	299.0	296.6	296.2	299.0	303.1	297.5	299.1	297.6	297.3	299.6	299.6	302.1	302.1	313.1	300.5	303.0
m 野菜交付量 (g)					キャベツ 10 q							キャベツ 10 r							キャベツ 10 s					

A. 群

Table 9-2

a 実験月日 (1962)	16/II	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
b 実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
c 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
d ラット体重 (g) No. 1	294	288	298	295	300	298	297	302	300	298	307	302	302	303	304	301	304	309	303	311	303	310	306
No. 2	315	312	316	311	316	311	315	317	314	311	321	318	318	322	323	319	319	328	328	328	322	330	327
No. 3	300	295	303	301	304	300	305	308	311	312	317	320	314	318	318	315	316	327	329	326	317	321	323
No. 4	301	296	302	296	302	300	301	307	308	306	311	310	312	309	311	309	312	323	323	323	320	320	321
No. 5	295	291	299	294	301	306	304	308	308	307	307	311	312	314	315	309	308	320	319	318	315	319	320
No. 6	296	295	295	278	293	290	291	295	293	290	300	302	303	311	307	306	310	319	318	317	313	319	317
No. 7	305	303	306	284	295	294	298	299	302	304	301	303	304	308	311	304	310	317	315	315	307	313	315
No. 8	317	314	316	298	309	309	311	316	314	312	312	315	314	319	317	315	317	330	329	329	321	328	327
No. 9	322	320	324	305	314	317	319	321	318	315	317	325	321	330	327	324	325	334	333	335	331	335	336
No. 10	271	267	266	250	264	260	261	263	259	257	267	273	268	274	278	275	279	280	284	287	283	281	284
e 平均	301.6	298.1	302.5	291.2	299.8	298.5	300.2	303.6	302.6	301.2	306.0	307.9	306.8	310.8	316.1	307.7	310.0	318.7	318.1	318.9	313.2	317.6	317.6
f 野菜交付量 (g)		トゥ ゾ 10 n							トゥ ゾ 10 o							トゥ ゾ 10 p							トゥ ゾ 10 q
g 実験月日 (1962)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
h 実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
i 飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
j ラット体重 (g) No. 1	309	315	318	317	313	317	315	317	317	319	320	316	316	315	313	314	314	315	313	314	315	312	320
No. 2	322	334	331	332	329	331	331	330	338	337	335	336	333	334	330	331	332	332	331	332	332	328	337
No. 3	323	334	325	324	324	325	323	328	332	338	328	323	323	325	324	326	325	328	324	324	323	320	329
No. 4	321	323	321	324	323	324	322	325	328	328	329	324	323	324	320	322	318	323	324	316	317	319	322
No. 5	318	322	327	326	322	321	323	323	328	335	335	327	325	324	322	323	324	324	322	325	324	323	330
No. 6	325	329	334	333	330	334	331	326	335	339	331	337	334	338	336	334	338	336	336	335	334	333	340
No. 7	316	317	317	318	317	317	321	316	315	322	318	321	320	320	320	324	321	322	325	321	321	323	328
No. 8	327	325	327	331	329	334	331	332	336	336	332	330	332	334	330	336	335	334	335	331	333	332	337
No. 9	335	344	335	335	335	335	339	338	348	343	341	339	340	337	334	342	336	336	340	339	341	337	346
No. 10	283	285	294	296	291	296	297	293	293	298	295	293	299	296	293	294	299	299	298	299	298	297	300
k 平均	317.9	322.8	322.9	323.6	321.3	323.4	323.3	322.8	327.0	329.5	316.4	324.6	324.5	324.7	322.2	324.6	324.2	324.9	324.8	323.6	323.8	322.4	328.9
m 野菜交付量 (g)							トゥ ゾ 10 r							トゥ ゾ 10 s						トゥ ゾ 10 t			

Table 10

1 ラットの群別	2 番号	3 性別	4 体重 (g)	5 6 赤血球数	5 7 白血球数	8 血色素 (%)	9 色素指数	10 白血球百分比 B	E	N St	N S	L	Mo	Pl
11 無処置対照 I	No. 1	♂	304	725	8,900	85	.59	0	0	1	57	40	2	0
	No. 2	〃	378	835	9,300	96	.58	0	0	11	27	59	3	0
	No. 3	〃	332	795	7,500	85	.53	0	0	7	35	55	3	0
	No. 4	〃	286	860	8,400	88	.54	0	1	3	13	82	1	0
	No. 5	〃	300	810	9,700	83	.51	0	1	0	21	76	2	0
	No. 6	〃	324	735	7,500	89	.60	0	2	7	27	63	1	0
	No. 7	〃	389	750	8,300	90	.60	0	2	8	33	51	6	0
	No. 8	〃	312	790	6,900	83	.53	0	3	10	35	49	3	0
	No. 9	〃	339	820	6,900	85	.52	0	1	9	34	56	0	0
	No. 10	〃	295	845	8,100	92	.54	0	1	10	28	60	1	0
12	平均		325.9	796	8,150	88	.55	0	1.1	6.6	31.0	59.1	2.2	0
II C.P. 1 %	No. 1	♂	315	735	12,300	79	.52	1	1	11	4	78	5	0
	No. 2	〃	340	765	4,400	90	.59	0	4	20	12	58	6	0
	No. 3	〃	311	695	6,700	85	.61	0	1	22	9	61	7	0
	No. 4	〃	336	970	8,100	86	.44	0	0	13	5	72	10	0
	No. 5	〃	341	740	7,300	87	.59	0	1	11	21	67	0	0
	No. 6	〃	328	785	6,800	85	.54	0	1	24	13	60	2	0
	No. 7	〃	364	670	7,600	89	.66	1	1	16	12	63	7	0
	No. 8	〃	353	705	7,800	78	.55	0	1	18	7	68	6	0
	No. 9	〃	371	715	6,600	82	.57	0	0	21	25	50	4	0
	No. 10	〃	329	760	8,400	89	.58	0	0	17	8	70	5	0
13	平均		338.8	756	7,600	85	.57	0.2	1.0	17.3	11.6	64.7	5.2	0
III C.P. 3 %	No. 1	♂	323	725	7,900	92	.63	0	1	12	6	70	11	0
	No. 2	〃	358	770	6,700	93	.65	0	0	10	7	83	0	0
	No. 3	〃	353	920	8,300	93	.50	0	0	21	6	64	9	0
	No. 4	〃	379	780	6,900	88	.56	0	0	13	5	78	4	0
	No. 5	〃	320	960	9,400	100	.52	0	0	14	8	73	5	0
	No. 6	〃	376	865	9,000	99	.85	0	0	14	7	76	3	0
	No. 7	〃	338	840	9,300	96	.57	0	3	14	5	73	5	0
	No. 8	〃	332	920	12,800	100	.54	0	0	8	6	76	10	0
	No. 9	〃	337	925	7,700	92	.50	0	3	11	3	77	6	0
	No. 10	〃	306	865	7,900	94	.58	0	4	9	2	77	8	0
14	平均		342.2	817	8,890	95	.59	0	1.1	12.6	5.5	74.7	6.1	0
IV C.P. 6 %	No. 1	♂	365	840	9,200	94	.56	0	3	11	9	72	5	0
	No. 2	〃	354	815	10,200	82	.67	0	0	19	4	73	4	0
	No. 3	〃	336	745	6,300	86	.79	1	2	12	3	79	3	0
	No. 4	〃	303	725	5,800	83	.57	1	5	13	7	71	3	0
	No. 5	〃	334	770	7,500	82	.62	0	1	14	12	66	7	0
	No. 6	〃	332	820	8,900	93	.75	0	2	5	5	83	5	0
	No. 7	〃	371	725	11,000	92	.63	0	1	14	4	77	4	0
	No. 8	〃	354	700	10,300	86	.61	0	1	23	9	56	11	0
	No. 9	〃	330	880	7,200	87	.64	2	0	11	12	72	3	0
	No. 10	〃	299	880	6,900	93	.70	0	0	14	6	80	0	0
15	平均		337.8	778	8,330	88	.65	0.4	1.5	13.6	7.1	72.9	4.5	0
V. S.P. 1 %	No. 1	♂	358	800	7,100	91	.57	0	2	20	5	70	3	0
	No. 2	〃	317	850	6,500	95	.56	0	1	17	1	73	8	0
	No. 3	〃	336	780	7,600	92	.60	0	2	22	4	62	10	0
	No. 4	〃	366	885	5,600	87	.69	1	2	26	11	54	6	0
	No. 5	〃	342	755	5,800	80	.52	1	0	20	9	65	5	0
	No. 6	〃	360	865	6,400	81	.50	0	11	17	1	66	5	0
	No. 7	〃	369	795	5,800	86	.61	0	1	11	9	78	1	0
	No. 8	〃	376	820	5,000	80	.65	0	1	20	8	70	1	0
	No. 9	〃	305	845	6,900	82	.43	0	1	20	1	57	21	0
	No. 10	〃	356	820	5,400	87	.70	0	0	13	3	76	8	0
16	平均		348.5	772	6,210	86	.58	0.2	2.1	18.6	5.2	67.1	6.8	0

Continuation of table 10

1 ラットの群別	2 番号	3 性別	4 体重 (g)	5 血球数 6 赤血球数	7 白血球数	8 ヘモグロビン %	9 色素指数	10 白血球百分比 B	E	N St	N S	L	Mo	Pl
VI S.P. 3 %	No. 1	♂	326	605	9,100	82	.72	0	0	13	6	68	13	0
	No. 2	〃	348	635	8,500	81	.64	0	0	23	13	63	1	0
	No. 3	〃	358	630	7,200	85	.68	0	1	28	4	60	7	0
	No. 4	〃	350	580	8,600	84	.72	0	2	13	3	76	6	0
	No. 5	〃	343	650	7,300	86	.66	0	0	13	8	77	2	0
	No. 6	〃	400	845	8,700	97	.57	0	0	20	7	68	5	0
	No. 7	〃	367	845	10,300	99	.58	0	0	26	12	58	4	0
	No. 8	〃	403	680	7,200	92	.68	0	0	10	9	73	8	0
	No. 9	〃	337	820	7,800	97	.57	0	0	20	8	71	1	0
	No. 10	〃	359	780	9,800	97	.62	0	6	23	16	54	1	0
	11 平均		359.1	707	8.450	90	.64	0	0.9	18.9	8.6	66.8	4.8	0
VII S.P. 6 %	No. 1	♂	320	620	8,700	91	.73	0	6	10	8	73	3	0
	No. 2	〃	337	690	7,800	94	.68	0	1	2	34	56	7	0
	No. 3	〃	329	740	9,900	95	.64	0	2	7	18	67	6	0
	No. 4	〃	322	785	10,400	100	.64	0	4	17	31	47	1	0
	No. 5	〃	330	690	7,600	98	.71	0	2	7	12	72	7	0
	No. 6	〃	340	685	7,600	93	.68	1	5	12	17	58	7	0
	No. 7	〃	328	655	7,200	89	.68	0	2	9	13	70	6	0
	No. 8	〃	337	730	5,600	90	.62	0	0	14	16	65	5	0
	No. 9	〃	346	855	6,100	96	.56	0	1	6	5	84	4	0
	No. 10	〃	300	760	5,200	95	.62	0	5	18	9	62	6	0
	12 平均		328.9	721	7,610	94	.66	0.1	2.8	10.2	16.3	65.4	5.2	0

Table 11

1 ラットの群別	2 番号	3 性別	4 体重 (g)	5 大脳 重量 (g)	6 大脳 容積 (ml)	8 小脳 重量 (g)	7 小脳 容積 (ml)	11 甲状腺 重量 (g)	9 甲状腺 容積 (ml)	14 胃 重量 (g)	10 胃 容積 (ml)	17 心臓 重量 (g)	12 心臓 容積 (ml)	20 肝臓 重量 (g)	13 肝臓 容積 (ml)	23 脾臓 重量 (g)	15 脾臓 容積 (ml)	26 腎臓 27 右 重量 (g)	16 腎臓 29 右 容積 (ml)	28 腎臓 31 左 重量 (g)	18 腎臓 32 左 容積 (ml)	33 副腎 34 右 重量 (g)	19 副腎 36 右 容積 (ml)	35 副腎 38 左 重量 (g)	21 副腎 39 左 容積 (ml)	40 睾丸 41 右 重量 (g)	22 睾丸 43 右 容積 (ml)	42 睾丸 45 左 重量 (g)	24 睾丸 46 左 容積 (ml)	47 肺 重量 (g)
49 I 無処置	No. 1	♂	304	1.27	1.23	0.54	0.53	0.04	0.02	1.54	1.48	1.15	1.09	10.3	9.5	1.34	1.20	1.00	0.92	1.00	0.93	0.02	0.01	0.03	0.02	1.27	1.22	1.34	1.29	1.75
	No. 2	〃	378	1.34	1.27	0.50	0.45	0.04	0.02	1.91	1.84	1.60	1.54	13.6	12.7	0.96	0.91	1.47	1.41	1.45	1.40	0.02	0.01	0.02	0.01	1.36	1.32	1.18	1.14	2.62
	No. 3	〃	332	1.08	1.05	0.51	0.49	0.04	0.02	1.83	1.79	1.25	1.20	11.5	10.7	0.86	0.83	1.09	1.02	1.10	1.06	0.02	0.01	0.02	0.01	1.31	1.27	1.33	1.30	1.89
	No. 4	〃	286	1.12	1.08	0.46	0.42	0.04	0.02	1.60	1.52	1.29	1.24	11.0	10.3	0.80	0.76	1.06	1.02	1.03	1.00	0.03	0.02	0.02	0.01	1.12	1.08	1.14	1.12	1.90
	No. 5	〃	300	1.10	1.08	0.50	0.49	0.04	0.02	1.60	1.54	1.24	1.22	10.6	10.0	1.10	1.04	1.09	1.04	1.14	1.10	0.02	0.01	0.02	0.01	1.42	1.38	1.51	1.47	1.72
	No. 6	〃	324	1.32	1.30	0.41	0.40	0.04	0.02	2.00	1.93	1.22	1.20	10.0	9.3	0.65	0.63	1.24	1.18	1.18	1.14	0.02	0.01	0.02	0.01	1.26	1.23	1.28	1.26	1.84
	No. 7	〃	389	0.91	0.89	0.46	0.44	0.04	0.02	1.84	1.80	1.40	1.36	11.0	10.2	0.87	0.83	1.15	1.11	1.06	1.02	0.02	0.01	0.02	0.01	1.24	1.23	1.30	1.28	1.92
	No. 8	〃	312	1.22	1.19	0.44	0.42	0.04	0.02	1.60	1.58	1.12	1.05	10.0	9.0	0.69	0.67	1.07	1.01	0.98	0.92	0.02	0.01	0.02	0.01	1.12	1.00	1.18	1.10	1.88
	No. 9	〃	339	1.28	1.22	0.55	0.53	0.04	0.02	2.20	2.07	1.20	1.13	11.2	10.5	1.05	0.99	1.24	1.20	1.24	1.20	0.02	0.01	0.02	0.01	1.44	1.40	1.44	1.37	1.77
対照	No. 10	〃	295	1.24	1.19	0.20	0.19	0.04	0.02	1.55	1.54	1.13	1.08	8.7	8.1	0.97	0.93	1.00	0.96	0.97	0.93	0.02	0.01	0.02	0.01	1.39	1.32	1.40	1.34	1.80
50	平均		325.9	1.19	1.15	0.46	0.44	0.04	0.02	1.78	1.71	1.26	1.21	10.8	10.1	0.93	0.88	1.14	1.09	1.12	1.09	0.02	0.01	0.02	0.01	1.30	1.25	1.31	1.28	1.91
II C.P. 1%	No. 1	♂	315	1.31	1.25	0.35	0.33	0.04	0.02	2.00	1.97	1.24	1.21	10.0	9.3	1.05	1.02	1.24	1.18	1.17	1.13	0.02	0.01	0.02	0.01	1.13	1.09	1.11	1.07	2.20
	No. 2	〃	340	1.15	1.11	0.40	0.38	0.04	0.02	1.91	1.87	1.61	1.55	9.0	8.4	0.91	0.87	1.11	1.09	1.08	1.04	0.02	0.01	0.02	0.01	1.19	1.15	1.16	1.12	2.10
	No. 3	〃	311	1.20	1.17	0.54	0.52	0.04	0.02	1.75	1.73	1.35	1.28	11.3	10.5	0.82	8.77	1.09	1.03	1.14	1.08	0.02	0.01	0.02	0.01	1.40	1.35	1.42	1.37	1.60
	No. 4	〃	336	1.08	1.04	0.40	0.38	0.04	0.02	2.30	2.21	1.35	1.27	10.0	9.4	1.00	0.95	1.10	1.03	1.15	1.08	0.04	0.03	0.02	0.01	1.15	1.08	1.10	1.09	2.80
	No. 5	〃	341	1.24	1.20	0.52	0.50	0.04	0.02	2.20	1.98	1.50	1.46	11.2	10.4	0.94	0.87	1.35	1.29	1.34	1.27	0.02	0.01	0.03	0.02	1.31	1.25	1.35	1.25	3.20
	No. 6	〃	328	1.26	1.21	0.48	0.46	0.04	0.02	2.10	2.02	1.28	1.21	10.5	9.8	0.32	0.27	1.07	1.01	1.08	1.01	0.03	0.02	0.02	0.01	1.22	1.19	1.26	1.22	2.00
	No. 7	〃	364	1.20	1.14	0.43	0.41	0.04	0.02	1.55	1.52	1.36	1.30	10.4	9.8	2.30	2.22	1.10	1.04	1.15	1.09	0.02	0.01	0.02	0.01	1.30	1.26	1.29	1.24	2.20
	No. 8	〃	353	1.14	1.10	0.40	0.36	0.04	0.02	1.82	1.74	1.34	1.29	10.9	10.1	0.93	0.89	1.02	0.98	1.14	1.09	0.02	0.01	0.02	0.01	1.46	1.42	1.52	1.48	1.45
	No. 9	〃	371	1.27	1.22	0.55	0.53	0.04	0.02	1.98	1.94	1.60	1.52	11.2	10.5	0.93	0.89	1.28	1.22	1.27	1.20	0.02	0.01	0.02	0.01	1.58	1.54	1.60	1.56	2.50
	No. 10	〃	329	1.34	1.30	0.54	0.52	0.04	0.02	1.76	1.71	1.39	1.34	10.2	9.5	1.01	0.96	1.16	1.11	1.14	1.11	0.02	0.01	0.02	0.01	1.18	1.15	1.22	1.19	2.00
51	平均		338.8	1.22	1.17	0.46	0.44	0.04	0.02	1.94	1.87	1.40	1.34	10.5	9.8	1.02	0.98	1.15	1.10	1.17	1.11	0.02	0.01	0.02	0.01	1.30	1.25	1.30	1.26	2.21
III C.P. 3%	No. 1	♂	323	1.22	1.16	0.44	0.42	0.04	0.02	1.62	1.56	1.22	1.17	11.0	10.1	0.84	0.80	1.12	1.05	1.04	0.98	0.02	0.01	0.02	0.01	1.34	1.30	1.36	1.32	1.96
	No. 2	〃	358	1.20	1.16	0.53	0.51	0.04	0.02	1.88	1.84	1.30	1.27	11.5	10.7	0.80	0.76	1.22	1.16	1.23	1.18	0.02	0.01	0.02	0.01	1.38	1.34	1.60	1.55	1.90
	No. 3	〃	353	1.30	1.28	0.55	0.53	0.04	0.02	1.58	1.54	1.25	1.23	9.3	8.6	0.76	0.74	1.00	0.95	1.00	0.95	0.02	0.01	0.02	0.01	1.30	1.26	1.31	1.27	1.88
	No. 4	〃	379	1.32	1.27	0.46	0.44	0.04	0.02	2.20	2.14	1.38	1.32	13.2	12.4	0.98	0.93	1.56	1.49	1.56	1.49	0.02	0.01	0.02	0.01	1.56	1.51	1.54	1.49	4.20
	No. 5	〃	320	1.23	1.18	0.54	0.52	0.04	0.02	1.40	1.35	1.22	1.17	8.8	8.2	0.80	0.78	1.06	1.02	1.12	1.07	0.02	0.01	0.02	0.01	1.42	1.39	1.46	1.42	2.40
	No. 6	〃	376	1.20	1.14	0.43	0.42	0.04	0.02	2.00	1.96	1.28	1.19	15.0	14.0	1.07	1.02	1.37	1.32	1.29	1.24	0.03	0.02	0.02	0.01	1.46	1.42	1.45	1.40	2.40
	No. 7	〃	338	1.02	1.00	0.40	0.39	0.04	0.02	1.58	1.56	1.15	1.12	10.5	9.8	0.90	0.86	1.10	1.08	1.15	1.12	0.02	0.01	0.02	0.01	1.20	1.18	1.23	1.20	2.60
	No. 8	〃	332	1.30	1.26	0.24	0.23	0.04	0.02	1.42	1.40	1.10	1.08	11.2	10.5	0.80	0.78	0.97	0.93	0.98	0.94	0.02	0.01	0.02	0.01	1.36	1.32	1.34	1.30	2.50
	No. 9	〃	337	1.14	1.09	0.42	0.40	0.04	0.02	1.48	1.42	1.24	1.20	10.9	10.1	1.28	1.23	1.13	1.08	1.18	1.13	0.02	0.01	0.02	0.01	1.13	1.09	1.11	1.07	2.50
	No. 10	〃	306	1.14	1.12	0.45	0.43	0.04	0.02	1.50	1.46	1.15	1.11	10.5	9.9	1.20	1.15	1.06	1.02	1.13	1.09	0.03	0.02	0.02	0.01	1.24	1.20	1.25	1.21	2.80
52	平均		342.2	1.21	1.17	0.45	0.43	0.04	0.02	1.67	1.62	1.33	1.19	11.2	10.3	0.94	0.91	1.16	1.11	1.17	1.12	0.02	0.01	0.02	0.01	1.34	1.30	1.37	1.32	2.51

Table 11 (continued)

IV	No. 1	♂	365	1.24	1.18	0.54	0.52	0.04	0.02	1.74	1.71	1.52	1.46	13.4	12.5	1.00	0.97	1.26	1.22	1.34	1.30	0.02	0.01	0.02	0.01	1.28	1.24	1.28	1.24	3.20
	No. 2	♂	354	1.32	1.28	0.46	0.44	0.04	0.02	1.61	1.57	1.30	1.28	13.7	12.8	0.91	0.89	1.18	1.14	1.10	1.04	0.02	0.01	0.02	0.01	1.10	1.06	1.10	1.06	2.50
	No. 3	♂	336	1.24	1.19	0.48	0.46	0.04	0.02	1.65	1.61	1.29	1.25	12.2	11.3	1.09	1.03	1.20	1.16	1.19	1.15	0.02	0.01	0.02	0.01	1.31	1.27	1.40	1.36	2.80
	No. 4	♂	303	1.16	1.12	0.51	0.49	0.04	0.02	1.54	1.50	1.56	1.49	10.5	9.3	1.01	0.97	1.25	1.19	1.32	1.26	0.02	0.01	0.02	0.01	1.38	1.33	1.43	1.38	3.00
	No. 5	♂	334	1.15	1.13	0.54	0.53	0.04	0.02	1.78	1.76	1.32	1.29	12.0	11.2	0.78	0.75	1.24	1.21	1.20	1.15	0.02	0.01	0.02	0.01	1.54	1.51	1.50	1.47	3.80
C.P.	No. 6	♂	332	1.12	1.07	0.50	0.49	0.04	0.02	1.55	1.50	1.28	1.22	11.3	10.5	0.79	0.76	1.03	1.00	1.06	1.03	0.02	0.01	0.02	0.01	1.18	1.14	1.26	1.22	3.20
	No. 7	♂	371	1.31	1.27	0.46	0.44	0.04	0.02	2.20	2.15	1.40	1.35	12.9	12.1	1.15	1.10	1.35	1.29	1.30	1.24	0.02	0.01	0.02	0.01	1.66	1.61	1.60	1.55	3.20
	No. 8	♂	354	1.28	1.24	0.34	0.32	0.04	0.02	1.80	1.75	1.60	1.58	13.2	12.4	1.24	1.16	1.35	1.31	1.38	1.34	0.02	0.01	0.02	0.01	1.54	1.50	1.56	1.52	2.90
	No. 9	♂	330	1.15	1.12	0.38	0.36	0.04	0.02	1.76	1.73	1.33	1.29	11.9	11.0	1.04	1.02	1.24	1.19	1.16	1.11	0.02	0.01	0.02	0.01	1.26	1.22	1.29	1.25	2.40
6 %	No. 10	♂	299	1.24	1.19	0.50	0.48	0.04	0.02	1.52	1.49	1.24	1.18	10.2	9.5	0.70	0.68	1.06	1.01	1.12	1.07	0.03	0.02	0.02	0.01	1.14	1.09	1.15	1.11	2.40
53	平均		337.8	1.22	1.18	0.47	0.45	0.04	0.02	1.72	1.68	1.39	1.31	12.2	12.3	0.97	0.93	1.22	1.17	1.22	1.17	0.02	0.01	0.02	0.01	1.34	1.30	1.36	1.32	2.94
V	No. 1	♂	358	1.10	1.07	0.52	0.50	0.04	0.02	2.00	1.60	1.50	1.45	12.4	11.5	1.16	1.10	1.40	1.34	1.48	1.42	0.02	0.01	0.02	0.01	1.28	1.26	1.32	1.30	2.50
	No. 2	♂	317	1.40	1.34	0.55	0.53	0.04	0.02	0.87	0.82	1.25	1.21	10.2	9.3	1.08	1.04	1.25	1.18	1.20	1.15	0.03	0.02	0.03	0.02	1.52	1.49	1.55	1.51	2.00
	No. 3	♂	336	1.30	1.27	0.25	0.23	0.04	0.02	1.65	1.61	1.05	0.99	10.4	9.7	0.48	0.42	0.98	0.92	1.15	0.99	0.02	0.01	0.02	0.01	1.05	1.02	0.94	0.91	1.40
	No. 4	♂	366	1.30	1.23	0.25	0.22	0.04	0.02	2.00	1.93	1.40	1.32	12.8	11.9	1.08	1.05	1.25	1.19	1.25	1.19	0.02	0.01	0.02	0.01	1.18	1.13	1.20	1.15	2.00
	No. 5	♂	342	1.17	1.11	0.75	0.71	0.04	0.02	1.97	1.89	1.42	1.38	11.5	10.8	0.68	0.62	1.30	1.24	1.32	1.26	0.03	0.01	0.03	0.02	1.47	1.44	1.24	1.20	2.00
	No. 6	♂	360	1.34	1.30	0.52	0.50	0.04	0.02	1.70	1.68	1.27	1.23	10.8	10.0	0.65	0.63	1.18	1.13	1.35	1.30	0.02	0.02	0.02	0.01	1.22	1.18	1.22	1.18	2.00
S.P.	No. 7	♂	369	1.25	1.21	0.55	0.53	0.04	0.02	1.82	1.78	1.70	1.62	12.8	11.9	1.05	1.03	1.26	1.22	1.20	1.16	0.02	0.01	0.02	0.01	1.22	1.19	1.25	1.22	3.20
	No. 8	♂	376	1.30	1.27	0.50	0.48	0.04	0.02	1.76	1.73	1.30	1.25	11.5	10.7	0.74	0.72	1.18	1.16	1.25	1.23	0.03	0.02	0.02	0.01	1.35	1.32	1.44	1.41	2.30
	No. 9	♂	305	1.18	1.15	0.50	0.48	0.04	0.02	1.92	1.88	1.28	1.24	10.2	9.6	1.00	0.99	1.32	1.30	1.20	1.18	0.02	0.01	0.02	0.01	1.18	1.16	1.18	1.16	2.20
1 %	No. 10	♂	356	1.25	1.21	0.32	0.31	0.04	0.02	1.56	1.52	1.52	1.46	10.8	10.0	1.34	1.28	1.32	1.26	1.38	1.32	0.02	0.01	0.02	0.01	1.46	1.41	1.50	1.45	2.10
54	平均		348.5	1.26	1.22	0.47	0.45	0.04	0.02	1.73	1.64	1.37	1.32	11.3	10.5	0.93	0.89	1.24	1.19	1.28	1.22	0.02	0.01	0.02	0.01	1.15	1.26	1.28	1.25	2.17
VI	No. 1	♂	326	1.10	1.06	0.45	0.44	0.04	0.02	1.44	1.38	1.44	1.37	9.5	8.9	0.90	0.86	1.15	1.10	1.15	1.10	0.02	0.01	0.02	0.01	1.46	1.42	1.45	1.41	6.00
	No. 2	♂	348	1.27	1.22	0.55	0.53	0.04	0.02	1.76	1.70	1.40	1.36	12.8	11.9	0.84	0.81	1.35	1.30	1.34	1.29	0.02	0.01	0.02	0.01	1.42	1.38	1.45	1.37	2.00
	No. 3	♂	358	1.14	1.10	0.30	0.29	0.04	0.02	1.80	1.76	1.40	1.33	11.4	10.6	0.96	0.93	1.20	1.16	1.13	1.08	0.02	0.01	0.03	0.02	1.15	1.09	1.12	1.06	2.60
	No. 4	♂	350	1.10	1.05	0.38	0.37	0.04	0.02	1.52	1.45	1.29	1.22	11.1	10.9	0.50	0.46	1.22	1.17	1.18	1.13	0.02	0.01	0.02	0.01	1.25	1.20	1.20	1.15	1.50
	No. 5	♂	343	1.22	1.17	0.42	0.40	0.04	0.02	1.80	1.78	1.30	1.28	12.0	11.3	0.98	0.93	1.15	1.10	1.20	1.16	0.03	0.02	0.03	0.02	1.35	1.31	1.34	1.30	2.00
	No. 6	♂	400	1.17	1.16	0.42	0.40	0.04	0.02	1.86	1.79	1.20	1.12	13.4	12.6	1.16	1.13	1.10	1.04	1.20	1.14	0.03	0.02	0.03	0.02	1.40	1.36	1.36	1.32	2.00
S.P.	No. 7	♂	367	1.26	1.21	0.45	0.43	0.04	0.02	1.92	1.89	1.35	1.28	14.6	13.6	0.75	0.71	1.35	1.30	1.32	1.27	0.02	0.01	0.02	0.01	0.75	0.70	0.74	0.69	3.90
	No. 8	♂	403	1.28	1.24	0.51	0.49	0.04	0.02	2.00	1.94	1.48	1.41	14.4	13.6	0.89	0.87	1.36	1.30	1.42	1.35	0.03	0.02	0.02	0.01	1.34	1.30	1.38	1.34	2.50
	No. 9	♂	337	1.22	1.19	0.43	0.42	0.04	0.02	1.58	1.56	1.18	1.13	10.8	10.1	1.20	1.14	1.18	1.12	1.10	1.04	0.03	0.02	0.02	0.01	1.35	1.29	1.32	1.26	2.20
3 %	No. 10	♂	359	1.30	1.26	0.40	0.39	0.04	0.02	2.40	2.48	1.30	1.26	13.4	13.0	0.96	0.94	1.32	1.27	1.34	1.28	0.03	0.02	0.02	0.01	1.18	1.14	1.20	1.14	2.30
55	平均		359.1	1.21	1.17	0.43	0.42	0.04	0.02	1.81	1.77	1.33	1.28	12.3	11.6	0.91	0.88	1.24	1.19	1.24	1.18	0.03	0.02	0.02	0.01	1.27	1.22	1.26	1.20	2.70
VII	No. 1	♂	320	1.16	1.12	0.42	0.40	0.04	0.02	1.64	1.57	1.25	1.19	14.2	13.2	1.06	1.00	1.32	1.25	1.34	1.31	0.03	0.02	0.02	0.01	1.32	1.27	1.28	1.23	1.60
	No. 2	♂	337	1.14	1.10	0.48	0.47	0.04	0.02	1.84	1.79	1.28	1.22	12.0	11.2	0.65	0.61	1.16	1.12	1.16	1.12	0.02	0.01	0.02	0.01	1.15	1.10	1.19	1.14	2.00
	No. 3	♂	329	1.15	1.09	0.50	0.48	0.04	0.02	1.64	1.60	1.13	1.08	12.0	11.1	0.60	0.57	1.28	1.23	1.16	1.10	0.02	0.01	0.03	0.02	1.14	1.10	1.10	1.06	2.00
	No. 4	♂	322	1.24	1.18	0.40	0.38	0.04	0.02	1.74	1.72	1.28	1.22	12.2	11.3	0.74	0.68	1.36	1.29	1.37	1.30	0.02	0.01	0.02	0.01	1.72	1.66	1.72	1.66	2.40
	No. 5	♂	330	1.18	1.12	0.46	0.45	0.04	0.02	1.75	1.70	1.28	1.22	11.5	10.7	0.84	0.80	1.15	1.09	1.10	1.04	0.02	0.01	0.02	0.01	1.30	1.25	1.35	1.30	2.00
	No. 6	♂	340	1.26	1.21	0.51	0.49	0.04	0.02	1.70	1.67	1.36	1.29	11.0	10.2	0.95	0.88	1.23	1.17	1.35	1.29	0.02	0.01	0.02	0.01	1.29	1.24	1.30	1.26	2.50
S.P.	No. 7	♂	328	1.16	1.10	0.46	0.44	0.04	0.02	1.62	1.55	2.30	2.21	10.4	9.7	0.80	0.76	1.06	1.02	1.05	1.00	0.03	0.02	0.02	0.01	1.23	1.19	1.20	1.16	2.20
	No. 8	♂	337	1.20	1.16	0.47	0.45	0.04	0.02	2.20	2.14	1.58	1.52	11.5	10.6	0.82	0.77	1.52	1.45	1.46	1.39	0.02	0.01	0.02	0.01	1.25	1.21	1.27	1.23	2.00
	No. 9	♂	346	1.12	1.08	0.44	0.42	0.04	0.02	1.88	1.87	1.33	1.26	12.0	11.1	1.00	0.95	1.24	1.19	1.26	1.21	0.02	0.01	0.02	0.01	1.20	1.16	1.25	1.21	1.80
6 %	No. 10	♂	300	1.18	1.13	0.42	0.39	0.04	0.02	1.64	1.57	1.12	1.07	8.6	8.5	0.84	0.79	1.02	0.97	0.95	0.88	0.02	0.01	0.02	0.01	1.14	1.10	1.08	1.04	2.20
56	平均		328.9	1.18	1.13	0.46	0.44	0.04	0.02	1.77	1.72	1.40	1.33	11.5	10.8	0.83	0.73	1.23	1.18	1.22	1.16	0.02	0.01	0.02	0.01	1.27	1.23	1.27	1.23	2.07

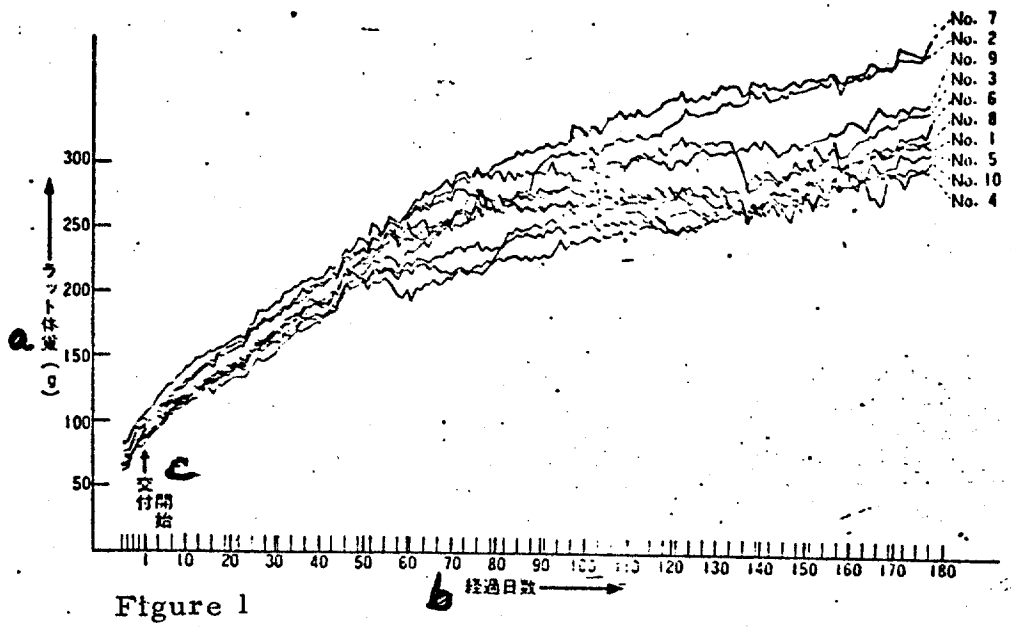

Figure 1

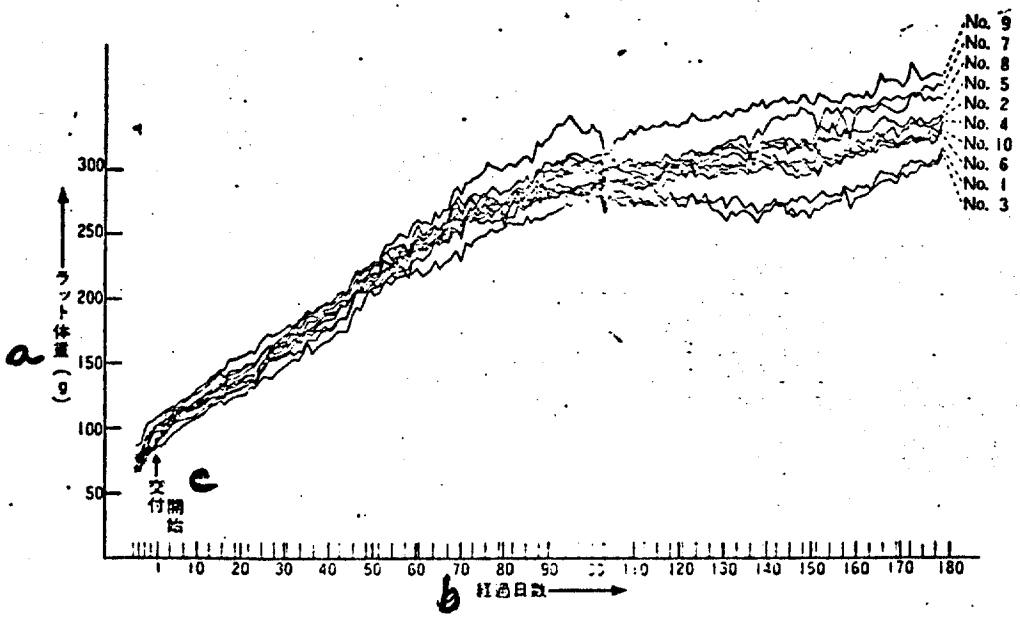

Figure 2

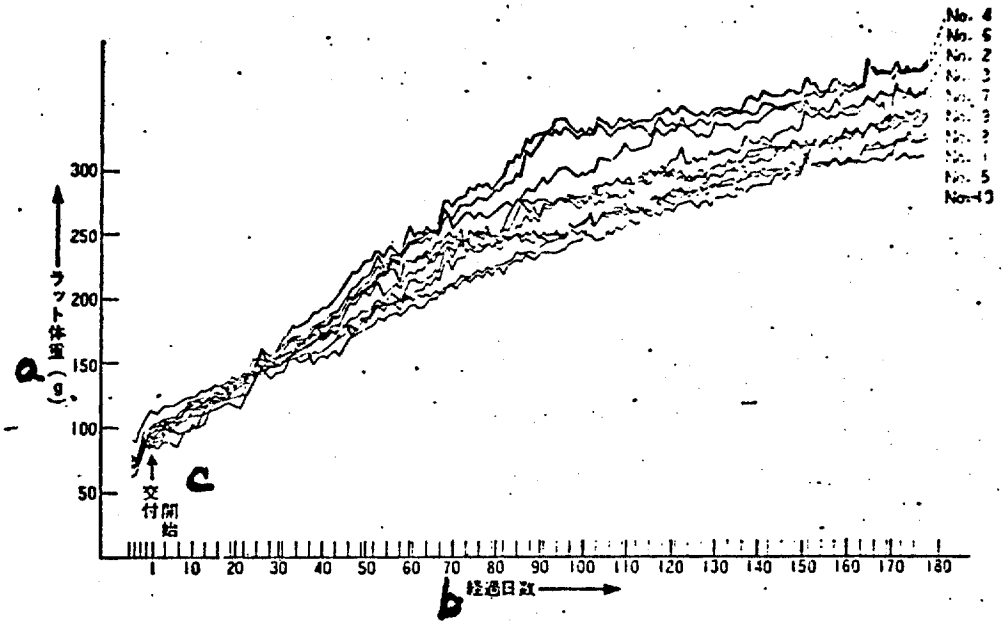

Figure 3

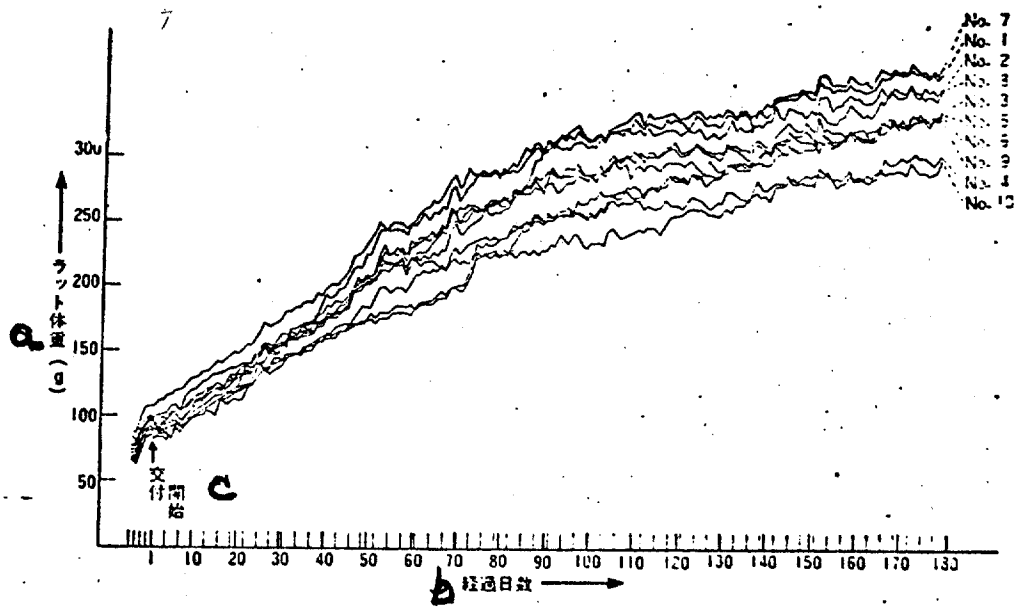

Figure 4

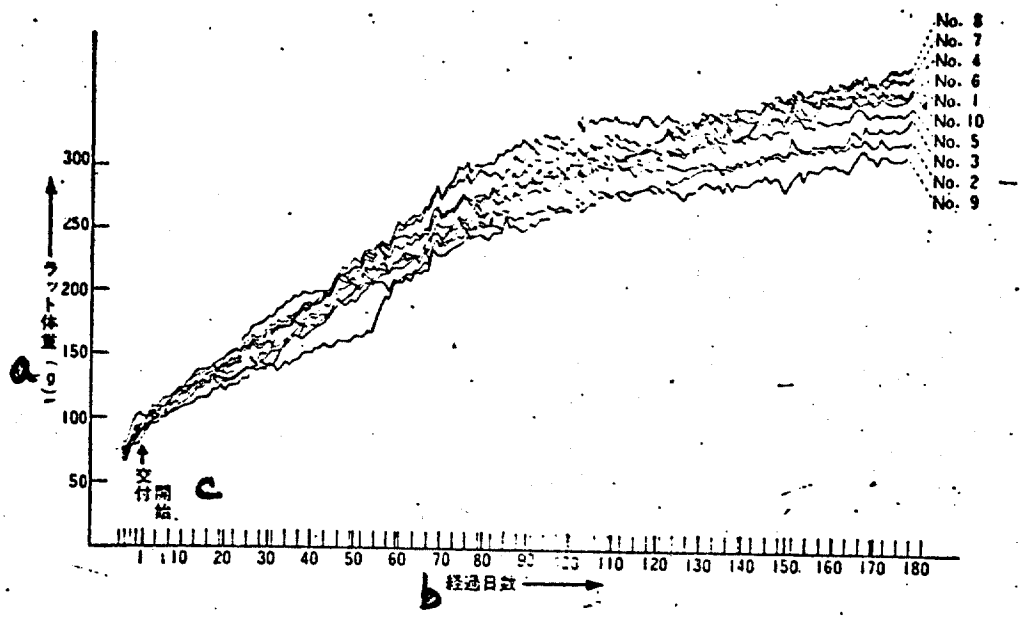

Figure 5

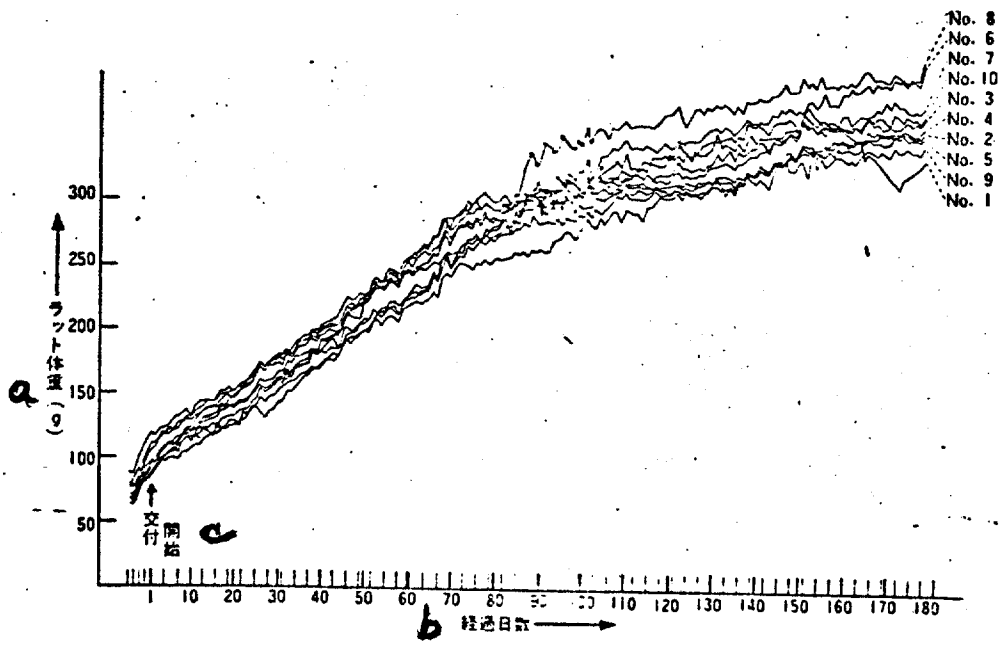

Figure 6

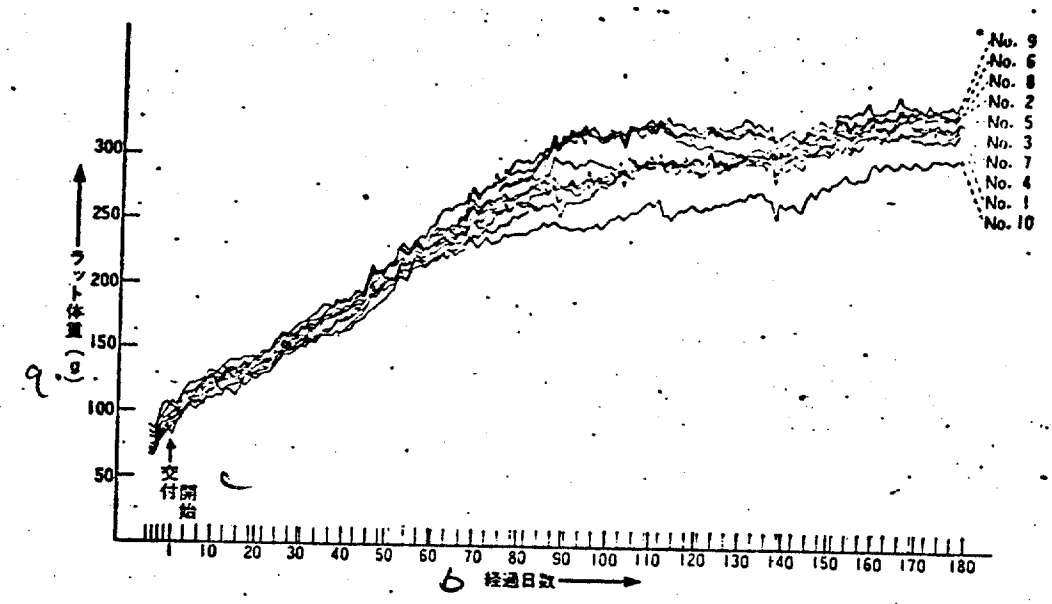

Figure 7

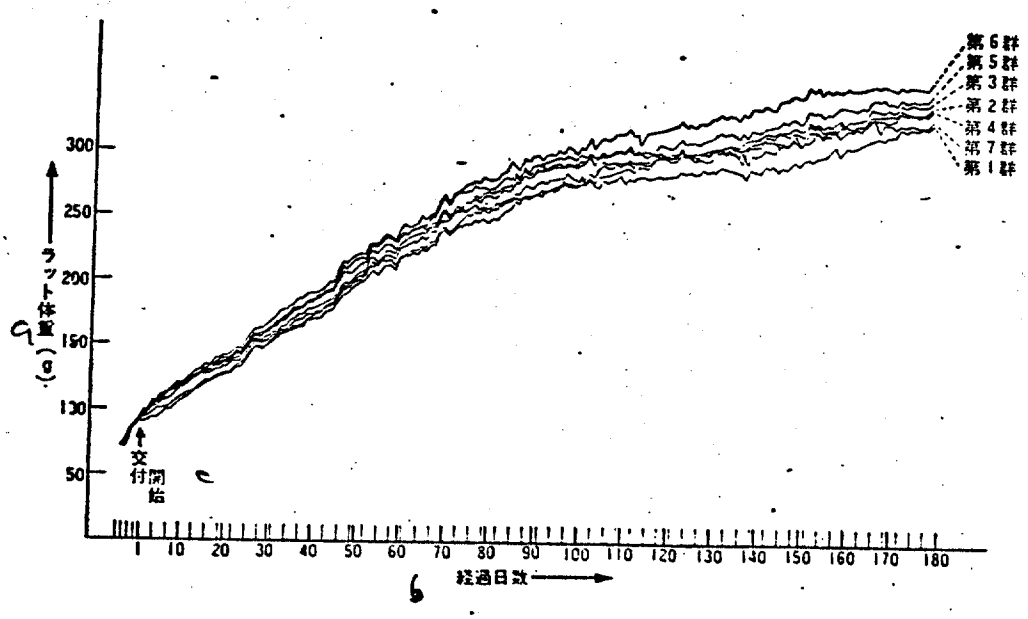

Figure 8

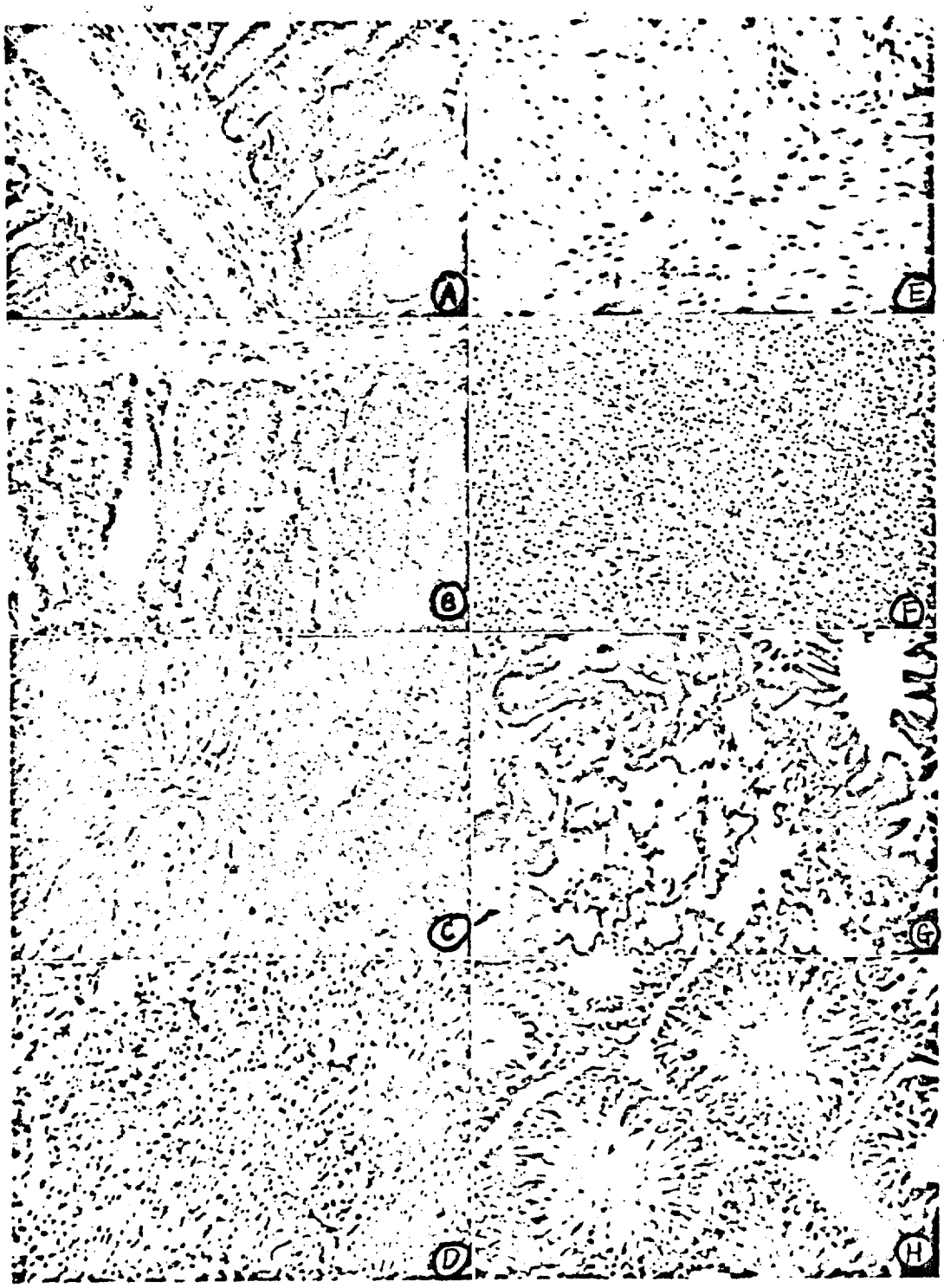

Figure 9

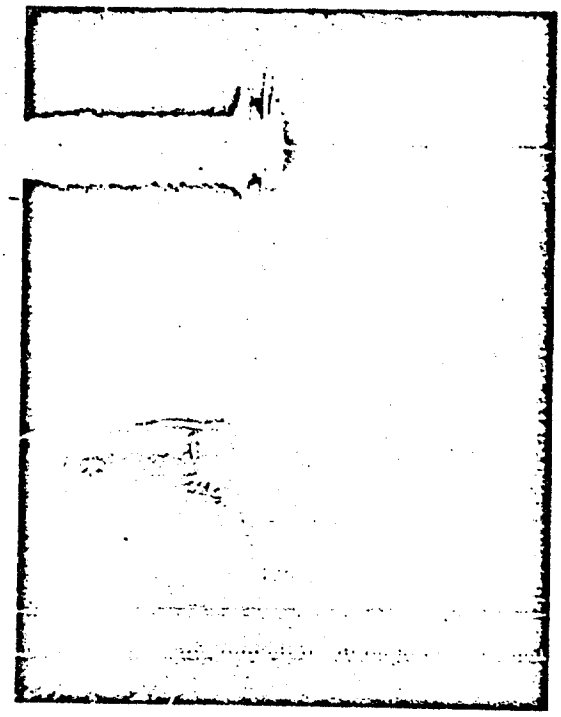

Figure 10

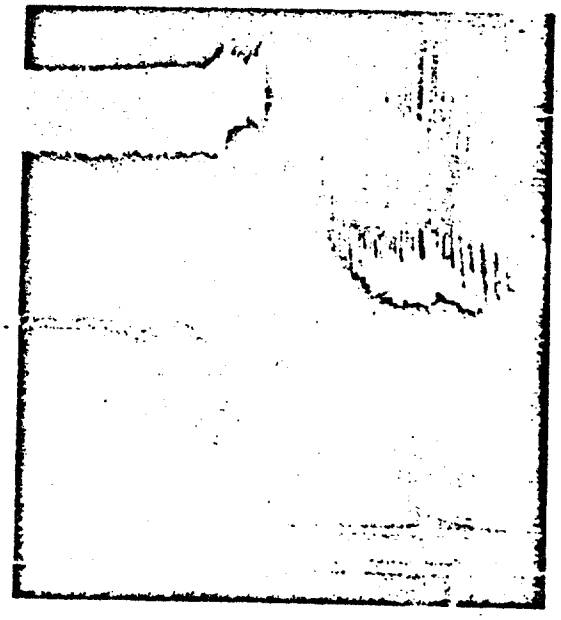

Figure 11

プロピオン酸塩の薬理作用および毒性の検索

Sodium Propionate 及び Calcium Propionate の一般薬理作用および毒性の検索

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時崎　謙

Studies of Pharmacological and Toxic Actions of Propionates

Examinations of General Pharmacological Actions and Toxicity of Sodium- and Calcium Propionates

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Department of Pharmacology, Tokyo Medical College
(Director: Prof. S. HARA)

Propionate is a substance which contains carbohydrates, organic acids and poly-valent alcohols as a basis and which is produced by fermentation with and aid of Bacterium propioni. It is either a colorless crystal or minute granular powder and has a faint but peculiar odor.

This substance is widely used in breads and cookies. An addition of this substance makes its end-products better, enhances mold-proof effect, prevents alteration of food value and thus, produces a beneficial influence in preservation of foods. It is therefore easily expected that propionate is used in our daily life as a substance to be added to foods as well as food preserve. Consequently, we think it necessary that this substance must thoroughly and rigidly tested with reference to its toxicity when used. For this very reason, we conducted these experiments in our laboratory.

Propionate showed practically no toxicity when given to mice by oral administration in our experiments of short duration. Propionate also showed no lowering of growth curve when administered to rats by mouth in experiments of long duration. It has no influence in hematological tests and in both weights and volumes of organs. We detected no pathological changes in histo-pathological examinations and experienced almost no detectable actions in our general pharmacological tests. In conclusion, we can safely state that propionate has on toxic property and can be used in foods as a substance to be added to a certain kind of foods.

I. 緒　言

近来，食生活の改善および進歩に伴つて主食品自体についてはもちろんのこと，食品添加物，食品保存物質，調味料および滋養品等に対する関心が著しくたかまつてきた．これらの物質は食品に添加されて，食品の鮮度を保持し，老化ないし変質を防止し，あるいは賦形等に効果および好影響をもたらすものである．

就中，プロピオン酸塩はパン，クッキイ等の製造に際して用いられ，その添加によつて仕上りを良好にし，ことに出来上つた食品の防微効果を著しく高め，また Kope 菌の発生ないし生育を抑制して食品の変質化を防止し，食品保存に良好なる影響を与えることが認められており，さらに動物の飼育飼料への添加に依

つて飼料保存に好影響を与えることも知られている．

プロピオン酸塩は炭水化物（ブドウ糖），有機酸（乳酸），多価アルコール等を基質として Propioni Bacterium 属に依り嫌気的条件下で酸酵し，その酸酵液をエーテル抽出または水蒸気蒸留に依り有機酸を集め，分留に依つて得たプロピオン酸に塩を加えて作られたものである．プロピオン酸塩には炭酸ナトリウムを加えたプロピオン酸ナトリウム（Sodium Propionate）および炭酸カルシウムを加えたプロピオン酸カルシウム（Calcium Propionate）その他がある．これら物質は白色の結晶性粉末あるいは粒状粉末で，わずかに特異の臭気を有する．本物質の溶解性は，Sodium Propionate は水に対し 10.8 mg/100 ml，Calcium Propionate は水に対し 3.6 g/100 ml である．

上記の知見は片桐，市川[1]のプロピオン酸酸酵に関する概説，工業技術院発酵研究所[2]の防微剤の抗菌力試験，農林省食糧研究所[3]のプロピオン酸塩の製パンおよび防微に及ぼす影響に関する報告，中江[4]の微とロープ，飼料事報の防微剤としてのプロピオン酸ソーダの問題，食品誌の総説，三共株式会社研究室の微及びロープ菌に対する抑制試験の報告書，RICHARDSON, L. R.[5]に依る飼料の昇温殊にその原因と防止，並びに日本イースト株式会社研究部の調査及び研究に於いて明らかにされ，余等も亦他の報告書等を併せ考察し，これを承認するところである．

併し乍らプロピオン酸塩は食品添加物乃至食品保存物質として日常使用されることが想定されるため，保健衛生上その毒性の有無を厳格に注意し検討せねばならない．

余等の教室に於いては，諸種動物，特にラットに対し薬物及び食品添加物の長期連続交付実験を行い，その一般状態，就中発育成長並びに体重に及ぼす影響を種々検索しており[6~15]，殊に被検物質の使用が比較的長期間に亘ると想定される場合に於いては充分なる毒性の検索を行う必要がある．

然るにプロピオン酸塩，殊に Sodium Propionate 及び Calcium Propionate に関しては長期に亘る毒性の検索なく，生理的乃至薬理学的作用に関する実験も見当らない．

茲に於いて余等は本物質の毒性の有無及び一般薬理作用を，毒性の発現に対し最も関係の深い成長曲線を中心として検索し，食品応用への参考資料として提供するため本実験を施行した次第である．

II. 実験材料並びに実験方法

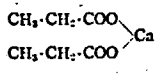
実験材料は日本イースト株式会社提供のプロピオン酸塩で，Sodium Propionate（以下 S.P. と略記）及び Calcium Propionate（以下 C.P. と略記）の2種である．その化学構造式は第1表の通りである（第1表）．

尚，実験動物はガマ，マウス，ラット，ウサギ等である．実験方法に就いては実験成績の各項で詳述する．

第1表　C.P. 及び S.P. の化学構造式　Table 1

C.P.

$$\left.\begin{array}{l} CH_3\cdot CH_2\cdot COO \\ CH_3\cdot CH_2\cdot COO \end{array}\right\rangle Ca$$



S.P.

$$CH_3\cdot CH_2\cdot COONa$$

III. 実験成績

〔A〕 毒性の有無に関する検索

1. C.P. 及び S.P. の急性毒性実験

15 g 内外の DD 系雄性マウスを用い，Behrens-Kaerber 法に従つて LD_{50} を求めると，第2表に示すごとくであつた（第2表）．

即ち，静脈内注射では C.P. は 580 mg/kg で，S.P. は 1,380 mg/kg であり，腹腔内交付では C.P. は 720 mg/kg で，S.P. は 2,250 mg/kg であり，皮下交付では，C.P. は 1,020 mg/kg で，S.P. は 3,200 mg/kg であつた．更に経口交付においては，C.P. は 3,340 mg/kg で，S.P. は 5,100 mg/kg であつた．殊に経口交付に於いては S.P. の 6,000 mg/kg 交付によりマウスは 5′ 後にやや運動減少し，次第に蹣跚歩行を行うようになり，15′ 前後には腹臓姿勢をとり，20′ 後には強制背位に応じ，ときに呼吸促迫を起す例も見られ，1h 20′ 前後に眼球突出及び白濁して斃死するのを認めた．また，C.P. の場合も 3,500 mg/kg 以上の量で，S.P. に於けると同様の経過をとり斃死する結果を得たが，両者共極めて大量を要した．

Table 2

第2表　マウスに於ける C.P. 及び S.P. の急性毒性（LD_{50}）表

被検物質 / 交付方法	C.P.	S.P.
静脈内交付	580 mg/kg	1,380 mg/kg
腹腔内交付	720 mg/kg	2,250 mg/kg
皮下交付	1,020 mg/kg	3,200 mg/kg
経口交付	3,340 mg/kg	5,100 mg/kg

第3表1　無処置対照ラットの体重推移表（g）

	実験月日 (1961)	30/IX	1/X	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
第I群	実験日数	1	2	3	4	5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
	飼育温度 (°C)	22	23	23	23	23	23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21
	ラット体重 (g) No. 1	81	83	90	99	102	105	107	112	118	121	127	127	133	136	140	141	146	148	148	152	152	154	156
	No. 2	82	83	94	94	93	99	100	106	108	111	117	118	121	127	130	130	134	138	140	143	140	150	149
	No. 3	76	76	85	92	101	100	103	109	111	112	118	120	123	124	126	131	137	139	141	145	144	151	152
	No. 4	74	72	84	88	95	100	102	109	110	110	115	116	114	114	116	118	124	126	127	129	131	137	136
	No. 5	73	70	82	86	88	88	91	96	99	99	105	103	110	112	113	114	116	118	121	124	119	127	126
	No. 6	68	64	76	84	86	89	90	98	103	104	112	113	113	115	116	120	124	124	125	133	133	135	136
	No. 7	67	67	77	84	90	96	98	104	106	105	113	114	113	118	117	122	126	128	130	131	130	138	134
	No. 8	62	64	74	82	84	87	96	93	96	97	103	108	112	113	113	117	121	122	127	130	129	131	133
	No. 9	67	68	75	80	88	93	93	99	103	100	113	115	115	117	117	118	125	127	130	132	132	136	138
	No. 10	64	68	76	79	79	83	88	92	94	98	102	117	107	110	111	114	119	121	122	128	128	131	137
	平均	71.4	71.5	81.3	86.8	90.6	94.0	95.8	101.8	104.8	105.7	112.5	115.1	116.1	118.6	119.9	122.5	127.2	129.1	131.1	134.7	133.8	139.0	139.7
	野菜交付量 (g)								キャベツ 10							キャベツ 10							キャベツ 10	
第II群	実験月日 (1961)	23	24	25	26	27	28	29	30	31	1/XI	2	3	4	5	6	7	8	9	10	11	12	13	14
	実験日数	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
	飼育温度 (°C)	21	21	21	22	23	23	23	23	23	23	22	23	23	23	21	22	22	22	22	22	22	21	21
	ラット体重 (g) No. 1	157	160	163	164	162	172	174	184	184	184	187	190	190	198	198	202	203	207	207	209	208	209	210
	No. 2	151	152	155	156	156	168	168	173	174	175	178	180	184	187	187	192	192	202	197	203	204	205	205
	No. 3	155	156	158	160	158	169	170	169	178	177	176	180	180	186	185	190	189	192	195	198	203	203	202
	No. 4	136	139	142	143	142	151	153	155	160	161	166	171	174	176	179	180	180	184	190	192	190	191	192
	No. 5	129	131	133	132	132	137	141	148	145	147	148	150	158	160	163	166	168	168	170	172	175	173	179
	No. 6	138	137	141	140	138	141	146	152	159	157	158	160	163	166	168	170	173	175	177	177	177	179	181
	No. 7	138	140	143	144	143	153	153	158	158	160	163	168	166	173	173	176	179	182	186	187	190	190	189
	No. 8	137	137	143	143	143	153	155	159	161	164	166	170	174	176	176	179	181	186	187	187	190	188	188
	No. 9	141	140	144	147	144	154	156	162	159	161	161	165	171	172	174	177	177	181	180	188	189	186	186
	No. 10	133	135	141	142	142	148	153	155	153	155	159	157	159	166	161	168	170	178	178	179	181	180	199
	平均	141.5	142.7	146.3	147.1	146.0	154.6	157.7	161.5	163.1	164.1	166.2	169.3	171.9	176.0	176.7	180.0	181.2	185.5	186.7	189.2	190.7	190.4	191.1
	野菜交付量 (g)						キャベツ 10							キャベツ 10							キャベツ 10			

第3表 1　無処置対照ラットの体重推移表（g）

第I群	実験月日（1961）	15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
	実験日数	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
	飼育温度（℃）	21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
ラット体重（g）	No. 1	218	216	218	227	231	228	232	240	239	234	251	249	243	251	257	253	252	254	257	260	262	263	266	264
	No. 2	210	213	210	224	226	224	228	228	231	228	241	239	237	242	247	241	238	250	252	242	240	238	239	242
	No. 3	208	208	208	222	223	223	226	230	232	232	241	243	241	250	253	251	247	255	258	263	269	268	275	273
	No. 4	197	199	197	210	215	216	218	222	223	225	233	230	234	241	244	239	237	244	247	247	248	253	254	240
	No. 5	183	182	184	200	205	200	202	203	206	206	214	215	213	217	215	210	207	215	215	212	218	219	220	216
	No. 6	183	187	185	197	205	206	206	212	211	216	222	221	221	225	225	223	216	225	230	229	231	235	237	232
	No. 7	189	198	199	209	217	211	218	220	226	231	240	239	241	245	253	251	247	255	254	258	265	260	264	260
	No. 8	190	196	199	209	212	210	214	208	209	200	206	205	206	209	205	199	194	193	149	190	201	199	204	203
	No. 9	189	194	194	211	215	215	213	218	220	220	225	227	231	235	236	248	232	239	239	238	242	244	248	248
	No. 10	180	189	190	194	199	200	202	203	206	208	212	212	213	214	217	213	212	216	221	217	223	223	222	220
	平均	194.7	198.2	198.4	210.3	214.8	213.3	216.4	218.4	220.3	220.0	228.5	228.0	228.0	232.9	235.2	232.8	229.2	234.6	237.2	236.5	239.9	240.2	242.9	239.8
	野菜交付量（g）				キャベツ10							キャベツ10							キャベツ10						

第I群	実験月日（1961）	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
	実験日数	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88
	飼育温度（℃）	22	22	22	22	22	22	22	23	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23
ラット体重（g）	No. 1	265	267	267	270	267	278	277	277	273	272	278	270	271	268	268	273	264	266	267	265	261	262	261
	No. 2	245	243	246	247	253	257	261	263	269	274	272	277	262	260	264	267	270	268	270	275	274	275	276
	No. 3	276	278	280	284	278	287	289	290	285	287	293	287	289	285	290	291	283	291	284	289	286	290	288
	No. 4	250	251	250	254	250	258	260	260	258	259	272	265	262	263	261	260	259	263	265	265	260	263	261
	No. 5	217	213	213	213	210	214	217	214	213	213	212	210	214	218	225	230	234	243	242	247	245	246	247
	No. 6	242	241	249	252	244	250	249	251	251	263	263	258	270	268	259	262	267	268	267	271	272	271	268
	No. 7	272	272	274	275	273	283	285	286	281	286	292	286	288	292	295	296	298	300	301	306	302	307	305
	No. 8	208	205	204	209	207	213	215	214	212	216	218	216	222	219	220	223	221	223	225	224	222	225	221
	No. 9	248	248	249	251	250	260	258	259	253	257	263	255	262	262	264	268	263	266	269	274	268	273	273
	No. 10	224	225	225	228	227	232	232	229	232	233	236	229	232	229	232	232	233	237	238	241	240	242	241
	平均	244.7	244.3	245.7	248.3	245.9	253.2	254.3	254.5	252.7	256.0	259.9	255.3	257.2	256.4	257.7	260.2	251.2	262.5	262.8	265.4	263.0	265.4	264.1
	野菜交付量（g）	キャベツ10							キャベツ10							キャベツ10							キャベツ10	

第3表 2　無処置対照ラットの体重推移表（g）

第I群	実験月日（1962）	1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	実験日数	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	204	105	106	107	108	109	110	111
	飼育温度（℃）	22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	22	21	22	22
ラット体重（g）	No. 1	262	263	260	264	265	265	264	263	268	268	264	267	263	265	259	254	261	260	261	262	265	257	258
	No. 2	289	293	297	300	303	301	303	304	306	303	303	305	303	309	303	303	310	311	311	315	316	313	314
	No. 3	289	290	288	289	298	288	289	287	290	284	289	294	293	302	290	288	293	296	297	297	296	292	299
	No. 4	260	261	259	262	265	265	263	264	268	262	266	272	269	274	268	265	272	267	264	266	270	263	273
	No. 5	249	247	248	251	253	250	255	257	262	257	258	258	259	265	263	266	271	271	267	265	271	265	265
	No. 6	269	271	269	272	275	275	275	277	278	274	272	272	273	275	269	266	277	270	269	272	277	273	269
	No. 7	305	308	308	312	308	310	312	311	313	326	324	324	317	324	325	318	325	330	330	334	337	330	335
	No. 8	224	223	225	224	225	230	238	229	233	237	234	235	237	240	239	235	241	238	241	241	244	234	244
	No. 9	269	278	275	274	273	274	275	277	283	283	284	283	286	296	279	282	286	285	289	292	295	290	296
	No. 10	240	242	239	245	243	247	247	242	247	247	246	250	249	253	247	245	249	248	250	247	252	249	251
	平均	265.6	267.6	266.1	269.3	270.8	270.5	272.1	278.1	274.8	274.1	274.0	282.2	274.9	280.2	274.2	272.0	278.5	277.6	277.9	279.1	282.3	276.6	280.4
	野菜交付量（g）						キャベツ10							キャベツ10							キャベツ10			

第I群	実験月日（1692）	24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	実験日数	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
	飼育温度（℃）	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
ラット体重（g）	No. 1	258	259	256	254	250	245	246	242	243	245	240	245	247	247	242	246	274	249	252	256	256	257	257
	No. 2	315	318	316	315	317	318	317	322	323	325	327	330	338	333	333	332	333	334	335	335	340	340	342
	No. 3	300	297	304	302	300	303	308	303	303	309	311	316	309	312	311	308	310	310	311	309	312	312	313
	No. 4	270	272	269	272	268	260	265	267	267	268	268	277	275	277	271	274	277	277	273	269	270	271	261
	No. 5	266	265	270	270	268	269	272	274	278	273	274	268	267	263	267	268	270	265	263	261	263	267	263
	No. 6	270	267	266	265	270	277	275	269	272	272	267	278	272	272	271	270	271	269	273	269	278	273	273
	No. 7	332	338	333	332	334	336	338	343	344	345	344	351	343	345	343	345	347	345	350	348	355	352	354
	No. 8	240	245	247	248	249	247	253	249	245	242	241	246	243	244	246	246	254	250	251	250	253	252	255
	No. 9	300	302	300	300	299	297	295	298	292	291	298	298	294	298	304	304	307	302	301	302	303	302	305
	No. 10	249	249	254	250	251	256	252	249	255	256	257	257	258	260	258	255	257	254	258	257	262	262	261
	平均	280.0	281.2	281.5	280.8	280.4	280.8	282.1	281.6	282.2	282.7	282.7	286.6	284.6	285.1	284.6	284.8	287.3	285.5	286.7	285.6	289.2	288.6	288.4
	野菜交付量（g）				キャベツ01							キャベツ10							キャベツ10					

第3表 2　無処置対照ラットの体重推移表(g)

第I群																								
実験月日(1962)		16/II	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
実験日数		135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
飼育温度(°C)		22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
ラット体重(g)	No. 1	258	256	262	257	264	262	260	263	267	273	271	270	274	275	274	274	278	280	280	280	281	283	292
	No. 2	340	340	310	342	352	346	345	350	313	314	344	347	345	351	348	350	350	351	353	353	356	354	354
	No. 3	305	297	290	272	274	274	279	283	282	282	277	286	282	290	296	290	292	287	309	303	296	297	303
	No. 4	264	267	266	249	265	256	264	254	258	265	261	260	251	249	265	257	270	267	262	271	268	266	275
	No. 5	259	256	254	248	257	259	255	265	265	268	264	263	262	267	267	266	270	273	258	268	275	271	275
	No. 6	274	272	273	278	284	287	281	288	288	284	281	280	285	287	288	286	292	299	289	293	293	202	304
	No. 7	355	354	347	349	358	356	354	357	351	353	352	352	354	356	358	353	358	359	359	358	360	359	363
	No. 8	256	257	262	255	264	260	261	271	271	273	270	272	270	270	276	272	268	281	277	278	275	278	280
	No. 9	303	306	306	304	309	312	305	313	310	305	300	303	304	308	309	305	308	309	312	312	213	214	318
	No. 10	258	258	258	252	253	257	253	258	259	263	253	261	262	260	267	263	269	264	261	261	233	262	270
	平均	287.2	286.3	285.8	280.6	288.0	286.9	285.7	290.2	289.4	291.0	287.3	289.4	288.9	291.9	294.8	291.6	295.5	297.0	296.0	297.7	298.0	298.6	302.9
野菜交付量(g)			キャベツ 10							キャベツ 10							キャベツ 10							キャベツ 10

第I群																								
実験月日(1962)		11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
実験日数		158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
飼育温度(°C)		22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
ラット体重(g)	No. 1	291	290	290	293	289	293	295	298	300	300	303	297	298	301	302	306	306	305	305	305	309	307	304
	No. 2	364	356	356	357	358	361	358	362	360	363	367	366	367	367	368	375	373	374	374	372	372	376	378
	No. 3	313	298	300	301	307	310	311	312	316	315	320	321	324	321	322	328	328	330	330	330	330	331	332
	No. 4	284	275	286	289	277	272	283	280	266	268	261	263	280	285	284	282	273	284	285	286	289	285	286
	No. 5	279	272	276	276	275	277	280	282	294	293	286	286	289	290	290	301	298	298	295	297	299	298	300
	No. 6	307	286	292	294	295	295	296	302	302	306	303	305	306	304	304	314	310	313	313	314	317	311	324
	No. 7	364	345	352	361	361	361	362	364	371	371	367	367	368	365	370	383	380	379	379	377	376	376	389
	No. 8	284	284	288	288	284	288	288	292	298	300	303	301	298	304	303	306	308	309	309	307	309	309	312
	No. 9	316	317	319	326	326	325	323	318	327	330	338	332	330	331	329	336	335	332	337	337	336	335	339
	No. 10	274	276	276	282	287	283	280	282	282	284	284	283	287	283	286	286	284	289	289	287	288	289	295
	平均	307.6	299.9	303.5	306.7	305.9	306.5	307.6	309.2	311.6	313.0	313.2	312.1	314.7	315.1	315.8	321.7	319.6	321.3	321.6	321.2	322.5	321.7	325.9
野菜交付量(g)								キャベツ 10							キャベツ 10							キャベツ 10		

第4表 1　1% Calcium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表(g)

第II群																								
実験月日(1961)		30/III	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
実験日数		1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
飼育温度(°C)		22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21
ラット体重(g)	No. 1	86	87	98	103	104	交付開始	108	109	114	115	116	119	122	123	126	126	128	132	137	138	143	138	143	144
	No. 2	79	79	90	96	97		102	103	106	109	113	113	119	121	126	130	132	134	139	140	144	143	153	153
	No. 3	76	78	88	93	98		103	101	112	113	115	115	115	114	116	117	118	122	126	127	130	129	132	136
	No. 4	77	75	85	89	100		102	101	107	107	107	111	112	116	120	119	121	126	126	129	132	134	140	141
	No. 5	77	73	82	87	97		97	97	104	105	104	108	110	112	114	117	120	121	127	127	133	132	138	138
	No. 6	67	67	77	85	81		86	85	91	94	96	99	101	103	105	107	109	113	118	118	121	117	121	123
	No. 7	68	68	78	84	90		93	95	101	106	109	111	113	113	115	116	116	121	122	123	123	123	126	127
	No. 8	70	69	77	82	87		90	93	101	102	107	110	114	113	115	117	120	119	123	123	125	125	127	129
	No. 9	69	72	80	80	84		92	93	102	106	111	113	114	116	119	121	124	127	129	131	134	133	134	135
	No. 10	66	70	73	79	83		88	91	97	101	104	105	106	107	108	110	113	115	118	120	124	124	132	131
	平均	73.5	73.8	82.8	87.8	92.1		96.1	96.8	103.5	105.8	108.2	110.4	112.6	113.8	116.4	118.0	120.1	123.0	126.5	127.6	130.9	129.8	134.6	135.7
野菜交付量(g)										キャベツ 10							キャベツ 10							キャベツ 10	

第II群																								
実験月日(1961)		23	24	25	26	27	28	29	30	31	1/IX	2	3	4	5	6	7	8	9	10	11	12	13	14
実験日数		19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
飼育温度(°C)		21	21	21	22	23	23	23	23	23	23	22	23	23	23	21	22	22	22	22	22	22	21	21
ラット体重(g)	No. 1	144	145	149	149	148	157	158	163	163	166	165	170	174	180	177	180	186	187	186	188	192	192	193
	No. 2	153	155	156	159	156	166	170	172	171	173	174	178	180	176	184	181	188	189	192	193	197	196	198
	No. 3	137	138	141	141	141	150	154	156	162	161	162	163	165	169	166	166	173	172	175	175	178	175	177
	No. 4	143	145	148	149	149	157	160	163	160	164	166	167	172	175	173	173	181	181	183	186	189	188	189
	No. 5	139	141	145	145	147	155	158	164	163	166	169	171	175	180	180	187	191	189	191	194	195	195	196
	No. 6	125	125	127	132	130	136	140	139	138	143	145	148	150	153	152	156	166	158	162	164	166	167	170
	No. 7	128	130	136	138	136	142	146	152	155	153	155	156	159	156	170	169	168	168	168	174	176	176	178
	No. 8	130	131	132	138	134	143	148	152	152	153	155	158	160	165	167	172	176	177	179	185	186	189	188
	No. 9	134	136	136	137	135	147	147	155	153	156	156	161	165	169	170	173	177	176	177	183	187	188	190
	No. 10	134	137	139	141	139	149	150	161	156	157	159	164	173	173	171	171	176	176	178	181	183	183	184
	平均	136.7	138.3	140.9	142.9	141.5	150.2	153.1	157.7	157.3	159.2	160.6	163.6	167.2	169.6	171.0	173.1	178.2	177.3	179.1	182.3	184.9	184.9	186.3
野菜交付量(g)							キャベツ 10							キャベツ 10							キャベツ 10			

第4表 1　1% Calcium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表 (g)

実験月日 (1961)	15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
実験日数	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
ラット体重 (g) No. 1	197	199	198	211	215	213	218	220	220	218	227	228	225	231	233	231	229	238	243	240	239	243	246	243
No. 2	204	205	204	216	220	218	222	223	226	224	236	237	228	240	242	243	234	245	248	244	247	250	257	253
No. 3	184	184	184	200	206	205	205	210	211	208	215	225	219	221	226	223	222	232	236	239	238	241	247	246
No. 4	195	194	194	208	214	213	212	218	219	220	231	229	227	233	237	237	234	240	247	242	243	246	250	248
No. 5	199	203	202	217	221	221	224	225	228	227	235	236	239	240	242	239	236	247	257	248	250	253	258	253
No. 6	174	174	174	188	194	191	202	208	203	204	210	215	218	216	221	222	215	228	232	230	232	235	244	241
No. 7	184	184	185	195	208	205	210	215	213	216	222	224	225	225	230	234	232	236	238	237	243	241	247	247
No. 8	193	193	198	202	207	208	206	208	209	208	211	213	211	215	216	218	218	220	223	219	221	223	229	223
No. 9	194	194	194	207	214	206	218	222	226	224	238	242	242	247	250	248	247	257	261	258	259	256	267	261
No. 10	193	194	195	206	213	213	213	218	217	218	229	228	229	228	236	242	233	243	249	247	247	250	257	252
平均	191.7	192.4	192.8	205.0	211.2	209.3	213.0	216.7	217.2	216.7	225.4	227.7	226.3	229.6	233.3	233.7	230.0	238.6	243.4	240.4	241.9	243.8	250.2	246.7
野菜交付量 (g)				キャベツ 10							キャベツ 10							キャベツ 10						

実験月日 (1961)	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
実験日数	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88
飼育温度 (°C)	22	22	22	22	22	22	22	23	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23
ラット体重 (g) No. 1	251	248	262	262	255	260	265	265	264	272	273	268	271	269	262	270	275	276	275	278	270	285	276
No. 2	258	260	273	274	262	275	279	273	269	271	279	272	275	274	274	280	283	283	281	287	280	292	284
No. 3	252	252	259	262	261	264	271	265	268	265	272	267	269	267	255	272	278	276	270	277	270	284	282
No. 4	253	252	263	265	259	264	268	269	269	268	271	273	273	272	274	273	277	282	274	281	274	287	281
No. 5	259	259	271	279	273	274	280	281	280	278	282	286	283	280	283	284	287	291	289	295	288	297	299
No. 6	244	243	252	252	250	263	259	263	258	258	262	258	260	260	262	262	262	272	268	272	274	279	273
No. 7	245	245	255	253	245	263	264	260	261	263	267	263	268	270	274	270	275	273	277	279	272	276	275
No. 8	228	230	232	237	229	243	240	245	247	247	251	253	251	253	255	258	257	258	257	263	258	262	261
No. 9	269	267	283	287	280	290	291	294	295	298	305	302	301	299	300	304	302	305	309	310	305	322	318
No. 10	254	255	270	270	264	273	277	269	275	281	278	278	276	272	274	274	282	285	284	289	285	299	297
平均	251.3	251.1	262.0	264.1	258.0	266.9	269.4	268.4	268.6	270.1	274.0	272.0	272.7	271.6	271.3	274.7	277.8	280.1	278.4	283.1	277.6	288.3	284.6
野菜交付量 (g)	キャベツ 10							キャベツ 10							キャベツ 10							キャベツ 10	

第4表 2　1% Calcium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表 (g)

実験月日 (1961)	1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
実験日数	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
飼育温度 (°C)	22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22
ラット体重 (g) No. 1	287	275	285	288	286	286	286	280	283	279	273	287	276	280	265	268	277	274	272	272	275	270	274
No. 2	298	298	303	306	302	310	311	312	310	308	307	308	309	311	297	303	308	302	304	306	308	301	300
No. 3	285	285	283	282	282	282	281	280	280	282	281	286	286	298	266	276	280	277	277	278	279	271	278
No. 4	290	291	292	295	294	299	295	300	304	300	296	300	296	299	285	291	290	291	290	290	297	292	294
No. 5	297	299	298	298	301	295	302	303	305	307	308	302	302	307	288	297	302	300	298	300	302	302	304
No. 6	276	282	278	278	283	277	283	284	287	284	286	290	287	289	265	276	284	282	283	280	287	284	286
No. 7	276	277	278	277	282	282	282	283	286	286	287	290	288	291	266	279	289	290	291	292	288	289	289
No. 8	262	265	265	267	270	274	271	274	279	279	280	284	286	284	266	277	283	281	288	288	287	284	285
No. 9	322	324	330	334	334	338	341	335	333	328	328	334	330	329	310	321	320	324	322	327	330	326	333
No. 10	305	305	302	304	306	306	309	308	306	305	306	306	304	306	289	301	301	301	298	297	298	298	297
平均	289.8	290.1	291.4	292.9	294.0	294.9	296.1	295.9	297.3	295.8	295.2	298.7	296.4	299.4	279.6	288.9	293.4	292.2	292.3	293.0	299.4	291.7	294.0
野菜交付量 (g)						キャベツ 10							キャベツ 10							キャベツ 10			

実験月日 (1961)	24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15
実験日数	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
飼育温度 (°C)	22	21	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22
ラット体重 (g) No. 1	272	272	272	270	275	270	275	276	270	271	270	280	274	274	273	274	273	275	277	268	276	278	275
No. 2	304	305	303	305	304	311	309	306	306	306	306	307	311	309	311	311	314	316	315	315	316	315	315
No. 3	273	274	273	279	279	275	282	276	275	277	276	281	272	273	280	271	268	267	263	259	267	262	265
No. 4	292	290	285	295	292	305	300	300	300	298	300	308	297	300	300	298	302	301	303	301	302	301	302
No. 5	302	302	303	303	300	302	302	302	302	304	302	304	303	305	306	308	312	310	314	316	319	315	319
No. 6	283	287	287	280	282	291	291	289	293	297	292	302	293	295	294	292	294	297	299	298	300	301	305
No. 7	287	289	291	290	294	298	296	302	307	310	310	314	311	313	310	312	315	317	317	319	321	321	324
No. 8	285	286	288	287	293	300	304	303	302	304	305	307	303	300	302	300	305	306	309	309	311	311	310
No. 9	330	330	333	332	333	336	333	334	332	335	335	342	339	337	337	337	340	338	342	343	340	344	345
No. 10	295	295	296	291	291	306	298	297	295	295	295	298	295	295	294	292	293	294	297	295	296	295	300
平均	292.3	293.0	293.1	293.2	294.3	299.4	300.0	298.5	298.2	299.7	299.1	304.3	299.8	300.1	300.7	299.5	301.6	302.1	303.6	302.3	304.8	304.3	306.0
野菜交付量 (g)				キャベツ 10							キャベツ 10							キャベツ 10					

第4表 2 1% Calcium Propionate 含有飼料をラットに連続日間経口交付せる場合の体重推移表 (g)

第II群

実験月日 (1961)	16/II	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
ラット体重 (g) No. 1	269	270	272	265	267	271	271	276	270	271	272	276	274	277	277	273	276	279	282	279	278	280	278
No. 2	311	312	312	312	316	319	316	319	319	319	310	316	315	320	320	313	320	310	325	320	336	325	325
No. 3	267	266	264	258	264	267	267	272	272	273	265	269	264	266	267	262	268	267	270	273	273	275	275
No. 4	302	298	296	297	300	301	299	301	303	305	300	302	296	302	304	297	300	292	305	303	305	306	304
No. 5	316	313	300	317	321	319	320	321	321	323	317	313	316	316	318	313	314	321	321	320	323	322	321
No. 6	301	310	304	308	310	309	308	310	304	299	295	295	292	295	294	295	300	295	305	305	301	305	303
No. 7	324	315	324	330	326	331	337	339	339	337	310	340	340	347	346	342	342	329	343	346	342	346	341
No. 8	305	307	312	312	315	315	316	317	315	315	323	322	317	321	322	317	324	332	331	328	331	331	341
No. 6	344	345	350	348	352	346	350	351	351	354	350	351	350	355	356	350	349	357	355	354	351	352	350
No. 10	299	294	302	304	305	303	302	304	304	305	302	303	304	304	307	304	304	313	308	308	306	310	308
平均	303.8	303.0	303.6	305.1	307.6	308.1	308.6	311.0	309.8	310.1	307.4	308.7	306.8	310.3	311.1	306.6	309.7	309.8	314.5	318.6	314.6	315.2	314.6
野菜交付量 (g)			トキャベツ 10						トキャベツ 10							トキャベツ 10							トキャベツ 10

第III群

実験月日 (1961)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
ラット体重 (g) No. 1	284	268	276	277	279	280	282	286	289	290	291	290	288	297	292	297	296	303	302	302	306	304	315
No. 2	329	312	318	321	318	322	320	328	324	326	329	332	323	330	330	335	335	332	333	332	333	334	340
No. 3	287	283	282	286	285	286	283	292	298	296	296	295	292	302	301	306	305	306	305	301	303	302	311
No. 4	310	311	311	310	309	312	312	318	326	321	321	322	316	320	318	321	321	322	322	322	319	321	336
No. 5	330	327	330	328	328	328	328	334	338	340	336	336	333	332	334	340	335	339	335	335	338	338	341
No. 6	309	308	308	312	313	314	316	311	318	318	316	316	318	320	322	324	323	324	322	327	331	328	328
No. 7	346	346	342	346	347	347	347	348	352	354	352	354	355	354	355	358	358	358	363	362	360	365	364
No. 8	337	319	339	343	347	346	343	346	350	348	346	341	343	344	347	358	355	358	352	355	352	354	353
No. 9	356	357	355	356	354	359	354	360	372	366	368	367	367	363	367	382	373	368	370	372	373	372	371
No. 10	314	313	312	318	314	313	312	318	322	320	320	320	318	318	321	325	320	321	323	322	326	323	329
平均	320.2	314.4	317.3	319.7	319.4	321.7	319.7	324.1	328.9	327.9	327.5	327.6	325.3	328.0	328.7	334.6	332.1	333.1	332.7	333.0	334.1	334.1	338.8
野菜交付量 (g)							トキャベツ 10							トキャベツ 10							トキャベツ 10		

第5表 1 3% Calcium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表 (g)

第II群

実験月日 (1961)	30/IX	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
実験日数	1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
飼育温度 (°C)	22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21
ラット体重 (g) No. 1	90	88	100	105	112	交	111	110	114	116	116	116	118	120	122	122	124	127	127	128	132	131	132	134
No. 2	77	75	86	96	98		101	102	104	103	98	96	93	95	95	102	95	107	112	119	118	119	120	120
No. 3	78	76	87	93	94	付	96	100	99	101	98	100	102	103	107	108	108	107	109	111	113	114	115	118
No. 4	73	74	85	91	96		102	96	106	107	107	106	111	113	116	115	120	117	120	122	125	124	131	127
No. 5	75	75	82	87	88	開	92	90	93	101	101	105	106	112	119	119	120	121	123	126	130	131	133	134
No. 6	70	70	78	85	93		92	95	97	100	103	103	105	110	111	112	112	112	117	116	119	117	120	121
No. 7	70	69	79	84	83		89	82	88	88	87	84	86	96	97	98	99	99	102	112	119	117	121	124
No. 8	71	70	79	83	93	始	90	92	94	97	98	101	102	103	109	110	114	113	116	117	120	120	127	128
No. 9	67	69	77	81	88		84	92	94	98	98	102	104	106	111	114	116	116	120	121	123	126	124	126
No. 10	62	65	74	88	85		88	88	90	97	95	101	103	104	109	112	114	114	117	120	123	121	130	129
平均	73.3	73.1	82.7	89.3	93.0		94.5	94.7	97.9	100.8	100.1	101.4	103.0	106.2	109.6	111.2	112.4	113.3	116.3	119.2	122.2	122.0	125.3	126.1
野菜交付量 (g)									トキャベツ 10							トキャベツ 10							トキャベツ 10	

第III群

実験月日 (1961)	23	24	25	26	27	28	29	30	31	1/XI	2	3	4	5	6	7	8	9	10	11	12	13	14
実験日数	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
飼育温度 (°C)	21	21	21	22	23	23	23	23	23	23	23	23	23	23	21	22	22	22	22	22	22	21	21
ラット体重 (g) No. 1	138	137	138	141	139	151	151	158	149	152	152	153	158	161	166	163	166	167	169	174	175	176	176
No. 2	122	122	123	124	124	133	136	146	139	136	139	141	140	148	152	150	149	151	152	156	155	156	160
No. 3	116	115	118	120	128	134	141	142	143	145	145	148	150	152	156	150	160	160	163	164	166	165	169
No. 4	131	129	134	134	131	145	146	160	155	154	156	162	169	170	177	175	177	178	181	187	187	189	190
No. 5	133	135	135	135	138	141	147	142	150	150	153	152	154	156	160	158	161	163	167	170	168	171	170
No. 6	126	127	129	137	131	139	144	147	146	146	150	154	154	155	160	161	163	165	167	176	176	177	177
No. 7	122	122	123	130	130	136	141	138	143	142	148	146	150	150	150	153	153	156	156	146	153	153	151
No. 8	129	130	133	133	131	136	141	145	141	146	145	148	148	152	155	156	159	161	161	161	166	169	167
No. 9	126	129	130	133	133	141	145	153	150	148	152	156	163	165	167	169	169	170	172	176	178	179	181
No. 10	132	131	137	138	138	141	147	157	154	154	155	158	163	165	168	166	168	170	173	176	178	165	172
平均	127.5	127.7	129.5	132.5	132.3	140.0	143.9	148.8	147.0	147.3	149.5	151.8	154.9	157.4	161.1	160.8	162.5	164.1	166.1	168.9	170.2	169.8	171.1
野菜交付量 (g)						トキャベツ 10							トキャベツ 10							トキャベツ 10			

第5表 1　3% Calcium Propionate 含有飼料をラットに連続 180 日間経口交付せる場合の体重推移表 (g)

第III群

実験月日 (1961)	15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
実験日数	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
飼育温度 (°C)	21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
ラット体重 (g) No. 1	182	182	184	192	199	197	202	201	203	207	211	216	216	217	220	219	208	226	226	225	228	230	231	231
No. 2	163	166	170	176	180	182	183	187	187	189	193	195	191	196	201	198	195	201	211	211	209	209	212	213
No. 3	171	170	176	186	195	196	199	203	203	200	207	210	200	203	206	208	204	213	219	215	219	218	224	224
No. 4	195	198	201	209	213	217	217	223	226	226	233	233	228	235	238	234	230	240	255	244	248	247	252	248
No. 5	173	168	168	170	174	176	171	172	174	196	177	182	180	183	184	186	184	186	191	192	190	190	193	196
No. 6	183	184	183	192	198	198	200	206	208	211	220	222	217	222	228	225	225	233	240	240	241	240	244	241
No. 7	155	157	153	156	166	168	170	182	178	183	190	192	187	192	192	192	188	200	197	202	203	203	208	206
No. 8	172	171	173	177	177	166	180	182	183	181	188	191	188	193	193	195	195	195	196	195	200	198	200	199
No. 9	185	187	188	198	200	205	207	211	214	216	221	231	220	226	230	232	229	235	241	242	242	244	247	247
No. 10	176	177	179	186	194	193	195	199	205	207	210	214	215	221	227	226	222	224	227	226	229	228	231	225
平均	175.5	176.0	177.5	184.2	189.6	189.8	192.4	196.6	198.1	200.4	205.0	208.6	204.2	208.8	212.2	211.5	208.0	215.3	220.3	219.2	220.9	220.6	224.5	223.0
野菜交付量 (g)				キャベツ 10							キャベツ 10							キャベツ 10						

実験月日 (1961)	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
実験日数	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88
飼育温度 (°C)	22	22	23	22	22	22	22	23	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23
ラット体重 (g) No. 1	241	240	243	249	245	244	250	232	250	246	248	248	254	239	238	233	237	241	243	248	239	243	240
No. 2	219	216	229	238	227	236	238	237	241	243	245	241	247	244	246	248	252	261	267	274	274	281	280
No. 3	226	231	231	238	235	234	241	242	237	235	239	237	239	239	243	240	250	247	257	262	272	267	262
No. 4	253	255	272	272	267	274	275	275	283	281	286	283	286	280	285	290	292	302	303	307	305	320	317
No. 5	195	200	200	207	208	203	210	212	209	213	214	211	219	213	219	220	215	222	225	227	225	230	229
No. 6	250	249	268	264	258	264	270	270	270	272	275	274	273	276	280	280	286	286	288	294	290	313	307
No. 7	213	209	222	227	218	227	227	229	236	232	233	235	238	236	240	239	244	244	243	254	252	262	255
No. 8	202	200	200	205	207	209	215	213	213	215	218	216	221	218	222	222	223	225	227	232	231	232	233
No. 9	249	245	258	263	251	255	259	264	264	258	264	261	260	263	265	267	269	272	268	274	272	273	270
No. 10	232	232	238	241	239	242	243	244	243	247	249	247	247	244	245	244	245	241	244	246	245	247	245
平均	228.0	227.7	236.5	240.4	235.5	238.8	242.8	241.8	244.6	244.2	247.1	245.3	248.4	245.2	248.3	248.3	251.3	254.1	256.5	261.8	260.5	266.8	263.8
野菜交付量 (g)	キャベツ 10							キャベツ 10							キャベツ 10							キャベツ 10	

第5表 2　3% Calcium Propionate 含有飼料をラットに連続 180 日間経口交付せる場合の体重推移表 (g)

第III群

実験月日 (1962)	1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
実験日数	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
飼育温度 (°C)	22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22
ラット体重 (g) No. 1	238	245	243	240	240	250	243	242	249	248	245	248	247	255	251	258	261	258	263	260	261	271	262
No. 2	280	287	291	285	290	292	297	298	296	294	290	293	292	295	294	305	303	303	303	307	309	317	311
No. 3	268	258	269	266	267	270	274	278	272	270	267	269	270	280	283	275	277	278	275	279	283	287	290
No. 4	323	324	325	326	328	335	335	336	331	329	323	328	323	329	324	337	333	332	329	331	337	333	335
No. 5	227	230	232	231	231	231	233	234	238	240	240	241	246	245	240	242	242	248	245	251	249	251	257
No. 6	311	318	323	326	323	328	322	330	328	325	320	323	323	323	323	331	329	324	323	327	328	329	331
No. 7	260	256	270	269	268	271	273	270	274	276	269	273	269	277	268	273	270	275	275	276	274	275	281
No. 8	230	235	238	239	239	241	243	245	247	250	252	253	256	260	256	257	263	262	264	267	269	264	264
No. 9	270	273	273	272	270	270	273	273	277	274	275	275	275	280	275	283	284	286	284	289	285	286	286
No. 10	249	242	248	245	246	246	245	245	254	251	252	254	255	263	251	258	260	259	262	263	264	260	262
平均	265.6	266.8	271.2	269.9	270.2	273.4	273.3	274.5	276.6	275.7	273.3	275.7	275.6	280.7	276.3	281.9	282.2	282.5	280.3	285.0	285.7	287.3	289.9
野菜交付量 (g)						キャベツ 10							キャベツ 10							キャベツ 10			

実験月日 (1962)	24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15
実験日数	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
育飼温度 (°C)	22	21	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22
ラット体重 (g) No. 1	261	264	265	262	265	276	267	271	270	269	270	275	276	273	279	274	282	275	279	285	282	283	283
No. 2	311	312	312	313	314	329	324	322	324	327	325	336	330	327	325	321	323	321	321	332	330	330	330
No. 3	283	289	290	286	291	292	294	293	300	304	303	313	301	300	305	301	305	303	310	307	308	310	312
No. 4	331	333	333	333	333	339	338	340	343	338	340	345	342	340	338	337	340	336	339	342	343	342	342
No. 5	254	254	259	261	259	262	259	264	265	262	268	270	290	271	267	265	270	271	266	273	274	273	276
No. 6	328	329	329	327	320	333	337	331	330	331	330	331	328	330	330	334	334	330	331	339	340	337	337
No. 7	296	286	287	284	285	294	295	290	294	293	288	300	287	291	287	295	295	289	293	298	299	297	303
No. 8	264	266	265	267	272	274	275	272	277	280	278	281	275	282	280	279	284	285	291	291	289	291	295
No. 9	284	288	293	292	296	292	296	299	288	297	292	294	290	287	290	291	289	293	297	298	300	299	303
No. 10	258	263	262	264	262	270	273	274	277	274	275	281	277	278	278	274	280	280	283	281	284	286	287
平均	285.0	288.4	289.5	288.9	289.7	296.1	295.8	295.6	296.8	297.5	296.9	302.6	299.6	297.8	297.9	297.1	300.2	298.3	301.0	304.6	304.9	304.8	306.8
野菜交付量 (g)				キャベツ 10							キャベツ 10							キャベツ 10					

第5表 2　3% Calcium Propionate 含有飼料をラットに連続 180 日間経口交付せる場合の体重推移表 (g)

実験月日 (1962)	16/II	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
ラット体重 (g) No. 1	284	279	288	295	290	290	290	292	290	291	296	292	294	296	294	294	294	309	302	300	301	300	300
No. 2	329	327	329	332	332	332	332	336	338	341	343	342	342	344	346	339	339	349	342	343	339	343	340
No. 3	310	308	310	322	318	316	316	321	320	316	322	324	326	326	329	325	327	342	337	337	339	337	335
No. 4	341	340	340	350	352	350	348	356	355	353	353	353	353	356	356	354	356	368	360	362	354	354	355
No. 5	274	275	281	277	280	282	283	284	284	384	291	291	286	293	290	291	292	301	299	300	297	300	297
No. 6	339	339	339	341	339	338	340	346	346	346	345	342	344	345	346	347	348	353	353	356	352	351	357
No. 7	295	300	300	305	310	310	310	316	315	314	308	313	312	312	317	315	314	300	313	319	312	317	313
No. 8	295	294	298	297	302	300	302	303	302	303	306	311	310	315	319	314	314	318	316	316	313	316	317
No. 9	301	302	301	304	306	306	307	312	313	313	311	313	309	313	311	310	314	314	314	315	310	319	318
No. 10	289	285	287	287	290	294	292	295	295	295	296	296	296	299	301	296	298	301	298	299	300	300	298
平均	305.7	304.9	307.3	311.0	311.9	311.8	312.0	316.1	315.8	315.6	317.1	317.7	317.2	319.9	320.9	319.5	319.6	325.5	323.4	324.7	321.7	323.7	323.3
野菜交付量 (g)		キャベツ 10							キャベツ 10							キャベツ 10							キャベツ 10

実験月日 (1962)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
ラット体重 (g) No. 1	302	304	300	300	301	302	301	310	315	313	315	316	311	314	315	318	318	320	317	317	317	319	323
No. 2	342	350	344	342	344	344	343	346	352	348	350	355	354	352	353	358	355	354	354	350	355	353	358
No. 3	336	335	340	339	337	338	338	342	350	350	346	344	348	327	346	362	350	351	359	350	352	354	353
No. 4	364	365	360	360	358	362	358	359	377	374	368	371	371	369	371	378	369	375	371	373	371	374	379
No. 5	300	302	300	300	305	302	303	309	311	311	312	311	313	309	306	318	318	318	325	316	317	318	320
No. 6	354	357	351	357	355	357	356	362	381	374	371	369	369	370	365	372	370	370	371	371	371	371	376
No. 7	310	320	319	327	322	323	325	322	326	332	329	322	334	336	335	338	334	335	339	339	341	337	338
No. 8	316	317	320	321	319	322	328	320	326	330	330	331	332	337	331	337	334	340	339	333	337	332	332
No. 9	322	318	308	324	322	324	326	323	325	329	330	325	326	330	328	328	332	332	332	329	329	329	337
No. 10	303	303	298	301	303	303	302	300	301	303	304	303	302	307	306	302	305	308	304	305	305	306	306
平均	324.9	327.1	324.0	327.1	326.1	327.7	328.0	329.3	336.4	336.4	335.5	334.7	336.0	335.1	335.6	341.1	338.5	340.3	340.1	338.3	339.5	339.3	342.2
野菜交付量 (g)							キャベツ 10							キャベツ 10							キャベツ 10		

第6表 1　6% Calcium Propionate 含有飼料をラットに連続 180 日間経口交付せる場合の体重推移表 (g)

実験月日 (1961)	30/IX	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
実験日数	1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
飼育温度 (°C)	22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21
ラット体重 (g) No. 1	88	86	98	106	107	交付開始	107	110	112	115	116	119	120	121	128	129	130	133	134	137	141	141	143	146
No. 2	85	81	91	97	98		98	100	101	102	107	108	104	105	111	112	116	117	120	120	125	124	122	132
No. 3	77	77	89	94	99		94	91	91	94	95	101	95	99	104	108	110	113	115	117	121	121	120	124
No. 4	76	76	88	91	97		92	94	99	97	101	104	102	104	110	111	112	114	118	121	125	123	124	131
No. 5	71	71	80	88	88		89	89	88	87	93	94	93	97	103	107	107	112	115	117	121	119	126	126
No. 6	73	73	82	86	90		86	87	94	88	95	96	91	93	96	100	102	105	107	109	111	110	112	114
No. 7	67	69	79	84	91		89	91	95	96	102	105	105	114	118	119	121	126	127	130	132	132	133	136
No. 8	66	64	76	83	85		84	83	83	81	87	91	86	91	95	97	100	103	106	109	110	114	117	117
No. 9	67	67	76	82	83		83	84	88	86	82	92	93	95	97	98	99	100	104	103	99	113	109	111
No. 10	66	63	73	80	84		82	85	86	89	90	93	92	93	100	102	104	107	109	112	116	115	117	118
平均	73.6	72.7	83.2	89.1	92.2		90.4	91.4	93.7	93.5	96.8	100.3	98.1	101.2	106.2	108.3	100.1	113.0	115.5	117.5	120.1	121.2	122.3	125.5
野菜交付量 (g)									キャベツ 10							キャベツ 10							キャベツ 10	

実験月日 (1961)	23	24	25	26	27	28	29	30	31	1/IX	2	3	4	5	6	7	8	9	10	11	12	13	14
実験日数	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
飼育温度 (°C)	21	21	21	22	23	23	23	23	73	23	22	23	23	23	21	22	22	22	22	22	22	21	21
ラット体重 (g) No. 1	148	148	151	154	153	160	164	171	169	169	171	174	180	179	183	183	186	187	186	194	192	197	200
No. 2	132	133	137	137	137	141	145	152	150	150	152	153	154	160	158	160	164	164	163	168	168	175	176
No. 3	128	127	132	134	132	139	140	141	148	147	148	153	155	158	164	160	161	161	163	171	173	175	175
No. 4	127	132	133	138	136	140	150	156	151	152	154	157	151	166	166	169	168	165	169	173	170	179	176
No. 5	128	128	132	132	132	140	147	154	149	150	151	155	157	164	163	165	169	170	170	177	178	184	183
No. 6	116	118	120	124	122	127	132	135	138	140	140	143	145	146	148	146	152	154	156	159	161	161	163
No. 7	136	137	140	140	140	149	151	156	153	153	156	163	162	160	162	165	171	171	172	178	180	193	190
No. 8	120	122	133	140	141	146	150	154	146	150	147	153	155	158	158	165	165	165	163	169	171	176	176
No. 9	113	112	112	120	124	136	140	134	136	144	144	141	147	147	148	148	153	153	154	155	159	159	160
No. 10	118	118	121	123	123	129	133	136	132	134	136	139	145	145	147	146	149	150	150	157	156	163	161
平均	126.6	127.5	131.1	134.2	134.0	147.0	145.2	148.9	147.2	148.9	149.9	153.1	155.1	158.3	159.7	160.7	163.8	164.0	164.6	170.1	170.8	176.2	176.0
野菜交付量 (g)						キャベツ 10							キャベツ 10							キャベツ 10			

第6表 2.6% Calcium Proionate 含有飼料をラットに連続 180 日間経口交付せる場合の体重推移表 （g）

第IV群																									
実験月日（1961）		15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
実験日数		42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
飼育温度（°C）		21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
ラット体重（g）	No. 1	200	204	206	211	222	224	226	231	236	242	249	243	244	249	249	248	244	247	249	255	259	261	266	264
	No. 2	178	179	180	182	195	196	197	201	201	211	218	226	223	225	223	229	222	234	232	237	237	238	242	236
	No. 3	175	179	181	185	197	196	196	202	203	213	220	219	215	217	215	216	208	221	224	225	225	228	234	238
	No. 4	180	178	181	186	196	197	200	201	201	204	209	212	211	214	217	218	213	220	220	215	218	218	219	215
	No. 5	188	187	186	193	197	205	201	205	204	204	215	216	218	217	216	222	209	215	222	218	223	221	225	223
	No. 6	168	169	170	171	170	171	171	173	178	174	179	178	178	181	184	184	183	185	181	186	187	187	185	192
	No. 7	193	197	198	206	211	214	213	224	223	235	243	243	243	243	244	243	239	247	247	250	256	251	256	256
	No. 8	179	185	184	190	198	200	103	207	206	212	224	231	223	225	229	227	219	229	232	233	236	235	238	238
	No. 9	160	165	164	165	167	169	172	173	174	171	175	175	175	178	178	178	178	176	182	185	186	186	185	189
	No. 10	163	165	164	170	173	178	183	183	182	191	197	199	195	196	197	197	194	200	208	208	211	210	212	211
	平均	178.4	180.8	181.4	185.9	192.6	195.0	196.2	200.0	200.8	205.7	212.9	214.2	212.5	215.5	215.2	216.2	210.9	217.4	219.7	221.2	223.8	223.5	226.6	226.2
野菜交付量（g）					キャベツ 10							キャベツ 10							キャベツ 10						

第IV群																									
実験月日（1961）		9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
実験日数		66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	
飼育温度（°C）		22	22	22	22	22	21	22	22	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23	
ラット体重（g）	No. 1	269	275	282	285	275	278	291	286	285	288	287	284	289	283	290	287	289	288	291	298	301	306	306	
	No. 2	245	245	257	257	260	259	263	263	262	260	261	265	266	265	267	268	272	272	271	272	272	276	275	
	No. 3	242	240	245	250	250	250	252	258	257	258	263	261	266	262	262	264	269	270	274	279	277	283	278	
	No. 4	213	213	218	220	217	219	217	223	220	221	223	225	227	221	227	225	225	221	225	227	224	227	223	
	No. 5	228	226	244	249	240	240	247	249	250	253	260	255	257	256	258	260	270	274	271	278	275	285	282	
	No. 6	195	192	197	198	196	196	203	210	227	223	224	221	224	221	224	223	231	234	243	243	245	250	250	
	No. 7	262	258	274	276	266	272	278	280	286	286	285	286	289	286	287	290	295	291	299	307	303	310	310	
	No. 8	247	243	260	256	248	255	257	260	258	259	258	261	264	265	268	265	274	275	272	278	282	297	297	
	No. 9	189	190	198	198	200	200	207	217	233	228	233	231	232	235	236	240	242	243	247	248	247	255	255	
	No. 10	215	211	225	229	224	227	231	233	235	236	238	235	237	235	236	238	246	246	245	249	248	255	252	
	平均	230.5	229.3	240.0	241.8	237.6	239.6	244.6	247.8	251.3	251.2	253.2	252.4	255.1	252.9	255.5	256.0	261.3	261.4	263.8	267.9	267.4	274.4	272.8	
野菜交付量（g）		キャベツ 10							キャベツ 10							キャベツ 10							キャベツ 10		

第6表 2 6% Calcium Propionate 含有飼料をラットに連続 180 日間経口交付せる場合の体重推移表（g）

第IV群																									
実験月日（1962）		1/1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
実験日数		89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	
飼育温度（°C）		22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22	
ラット体重（g）	No. 1	302	305	308	308	306	307	306	310	311	310	307	308	307	314	310	319	322	323	324	326	330	333	333	
	No. 2	277	279	280	273	274	278	279	280	285	284	282	283	286	293	287	297	300	297	298	300	299	304	300	
	No. 3	271	283	292	287	282	291	290	290	294	286	285	286	286	290	287	293	297	296	295	301	298	296	303	
	No. 4	227	227	232	230	240	236	233	240	240	232	229	232	232	232	230	241	244	240	237	239	240	242	243	
	No. 5	283	288	288	284	285	285	285	284	290	285	286	285	280	288	284	288	297	292	291	294	282	291	293	
	No. 6	248	251	250	253	255	253	257	254	257	253	253	257	253	259	257	266	270	267	268	276	275	277	275	
	No. 7	304	313	310	312	316	312	316	313	320	315	315	313	313	316	311	317	318	318	316	321	321	329	324	
	No. 8	302	312	306	310	316	315	319	321	324	318	312	311	315	317	311	320	319	318	320	320	318	318	320	
	No. 9	250	253	253	254	258	258	261	256	266	263	263	268	267	274	269	270	270	272	273	275	270	267	269	
	No. 10	252	256	253	253	255	255	254	256	257	256	251	254	254	260	257	264	267	262	264	264	264	266	266	
	平均	271.6	276.7	277.2	276.4	278.7	279.0	280.0	280.4	284.4	280.2	278.3	279.7	279.3	284.3	280.3	287.5	290.4	288.5	288.6	291.6	289.7	292.3	292.6	
野菜交付量（g）							キャベツ 10							キャベツ 10							キャベツ 10				

第IV群																									
実験月日（1962）		24	25	26	27	28	29	30	31	1/2	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
実験日数		112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	
飼育温度（°C）		22	21	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22	
ラット体重（g）	No. 1	323	333	329	330	330	332	333	333	328	330	330	334	335	335	328	332	330	333	331	335	334	330	335	
	No. 2	296	300	300	300	301	307	307	311	304	302	304	306	310	306	302	304	303	303	302	309	308	308	306	
	No. 3	299	303	304	299	301	304	306	306	300	302	303	306	308	306	304	300	301	298	300	308	307	306	304	
	No. 4	241	245	245	244	239	249	253	254	255	255	258	260	259	259	258	255	255	254	257	260	257	262	262	
	No. 5	289	293	293	290	291	295	295	296	295	292	294	297	292	290	286	298	292	288	288	290	294	290	290	
	No. 6	268	275	276	276	278	282	285	281	281	281	278	287	285	285	286	285	285	284	287	287	293	293	295	
	No. 7	321	326	324	323	320	328	326	328	327	326	320	326	332	330	324	324	325	323	325	332	331	329	332	
	No. 8	316	322	317	317	318	320	320	320	316	317	320	319	320	321	313	315	318	314	316	318	329	320	323	
	No. 9	271	275	277	273	276	277	282	285	282	283	280	286	284	285	277	280	282	283	287	292	293	291	295	
	No. 10	259	261	261	260	261	266	262	262	262	259	264	269	268	267	263	260	261	264	263	273	273	271	268	
	平均	288.3	293.3	292.6	291.2	291.5	296.0	296.9	297.6	295.0	294.7	295.1	298.8	299.3	298.4	294.1	295.3	295.2	294.4	295.6	300.4	301.9	300.3	301.0	
野菜交付量（g）					キャベツ 10							キャベツ 10							キャベツ 10						

第6表 2　6% Calcium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表（g）

第IV群

実験月日 (1962)	16/II	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
ラット体重 (g) No. 1	335	333	317	336	334	330	334	343	344	345	345	345	346	349	349	345	344	359	354	354	349	352	353
No. 2	305	303	310	312	309	307	310	317	320	323	326	324	320	320	326	321	319	336	330	327	326	325	330
No. 3	299	296	306	309	306	305	303	311	310	309	319	317	313	312	312	312	308	331	319	322	318	319	319
No. 4	263	257	278	272	269	270	265	277	277	278	280	283	280	283	285	284	286	285	284	289	284	277	280
No. 5	290	290	294	295	295	291	294	300	302	304	309	306	304	304	306	306	306	313	310	313	310	309	311
No. 6	295	292	295	295	301	304	301	310	306	302	303	307	304	312	312	312	310	315	317	315	308	311	313
No. 7	333	331	332	334	336	340	338	344	345	347	346	348	348	352	350	350	348	362	364	361	355	356	356
No. 8	320	320	322	325	323	327	327	340	332	328	337	337	335	335	330	335	332	346	346	347	335	339	340
No. 9	293	294	297	300	303	303	305	310	311	313	312	316	316	317	320	316	312	303	314	315	312	312	312
No. 10	267	265	272	282	275	275	277	279	279	279	286	280	279	280	279	279	278	292	288	286	283	283	282
平均	300.0	298.1	301.5	306.0	305.1	305.5	305.4	313.1	312.6	312.8	316.3	316.3	314.5	316.5	316.9	316.0	314.3	324.2	322.6	322.9	318.0	318.3	139.6
野菜交付量 (g)		キャベツ 10							キャベツ 10							キャベツ 10							キャベツ 10

実験月日 (1962)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
ラット体重 (g) No. 1	352	356	352	352	351	351	352	354	362	361	362	366	365	366	365	368	365	365	363	361	365	360	365
No. 2	331	341	337	335	331	333	330	336	344	341	351	352	346	346	348	356	347	346	355	350	352	348	354
No. 3	317	319	319	315	313	316	313	318	328	325	327	330	326	325	322	326	329	334	328	329	332	326	336
No. 4	280	286	284	283	284	286	283	288	289	300	295	296	297	300	298	300	303	300	300	298	294	297	303
No. 5	314	320	313	317	313	313	312	314	323	328	330	320	322	324	324	327	327	329	329	327	330	327	334
No. 6	313	320	319	315	312	319	313	322	324	327	323	330	324	330	324	333	332	328	331	326	330	332	332
No. 7	355	363	360	358	357	358	353	362	368	367	364	368	365	364	364	374	369	366	366	363	367	363	371
No. 8	345	348	345	345	342	342	342	345	355	350	347	355	351	350	349	357	350	350	345	347	346	344	354
No. 9	317	318	320	320	322	319	320	322	319	320	324	326	325	328	324	331	332	330	327	323	330	327	330
No. 10	285	290	282	284	283	281	282	284	292	287	289	289	290	289	286	297	291	289	289	286	287	288	299
平均	320.9	326.1	323.1	322.4	320.8	321.8	320.0	324.5	330.4	330.6	331.2	334.1	331.1	332.2	330.4	336.9	334.5	333.4	333.3	331.0	333.3	331.2	337.8
野菜交付量 (g)							キャベツ 10							キャベツ 10							キャベツ 10		

第7表 1　1% Sodium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表（g）

第V群

実験月日 (1961)	30/IX	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
実験日数	1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
飼育温度 (°C)	22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	20	21	21
ラット体重 (g) No. 1	80	79	93	100	104	交付開始	100	101	105	106	108	111	115	116	121	122	127	130	136	136	138	137	141	134
No. 2	75	75	82	87	86		91	91	97	98	100	104	107	112	117	119	119	124	126	127	128	130	134	136
No. 3	76	77	84	92	93		97	99	103	107	110	111	113	118	123	121	128	130	133	133	134	137	141	143
No. 4	72	73	83	89	90		95	93	106	99	103	106	108	112	117	117	118	125	127	127	128	128	132	138
No. 5	71	70	80	82	89		94	94	102	104	107	108	110	112	117	118	122	123	125	127	132	134	136	138
No. 6	70	72	81	85	90		94	93	102	104	106	109	111	117	120	120	122	123	127	127	129	128	133	131
No. 7	74	72	81	84	93		96	102	109	107	112	116	116	119	123	122	124	124	126	131	133	134	136	138
No. 8	68	66	76	82	87		92	93	98	100	107	107	109	110	112	113	116	119	121	124	125	126	130	130
No. 9	64	67	76	80	86		91	91	97	98	101	102	101	104	106	108	109	110	114	115	115	115	121	122
No. 10	72	70	77	79	79		88	90	96	95	100	100	104	107	111	111	115	117	121	122	124	125	135	125
平均	72.2	72.1	81.3	86.0	89.7		93.8	94.7	101.5	101.8	105.4	107.4	109.4	112.7	116.7	117.1	120.0	122.5	125.6	126.9	128.6	129.4	133.9	133.5
野菜交付量 (g)									キャベツ 10							キャベツ 10							キャベツ 10	

実験月日 (1961)	23	24	25	26	27	28	29	30	31	1/XI	2	3	4	5	6	7	8	9	10	11	12	13	14
実験日数	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
飼育温度 (°C)	21	21	21	22	23	23	23	23	23	23	22	13	23	23	21	22	22	22	22	22	22	21	21
ラット体重 (g) No. 1	146	147	150	151	155	164	164	171	172	174	175	179	178	184	186	190	191	195	194	198	197	196	198
No. 2	136	133	135	135	138	142	142	147	140	144	145	151	152	152	152	160	161	165	162	162	165	168	170
No. 3	146	147	150	152	151	158	159	165	161	163	167	172	172	176	172	179	180	182	183	189	187	189	189
No. 4	139	139	141	143	143	152	154	159	156	157	158	163	166	170	170	176	177	176	182	187	189	188	190
No. 5	140	142	144	146	146	154	156	162	159	162	162	166	168	171	171	176	174	178	180	184	184	187	187
No. 6	135	137	139	142	142	145	150	158	154	154	157	160	154	167	170	170	170	174	171	174	175	178	177
No. 7	141	141	146	145	147	155	154	161	160	161	165	169	168	172	170	173	179	179	182	185	185	188	190
No. 8	131	130	130	130	133	137	140	145	142	150	149	153	155	156	160	162	165	168	169	176	177	181	180
No. 9	125	122	125	125	125	131	130	139	137	138	138	143	140	150	150	158	161	164	163	171	170	172	170
No. 10	128	127	130	132	133	137	138	137	140	143	142	140	141	135	140	143	141	145	146	150	151	151	154
平均	136.7	136.5	139.0	140.1	141.3	147.5	150.7	154.4	152.1	154.6	155.8	159.6	159.4	163.3	164.7	168.1	169.9	172.6	173.2	177.6	178.0	179.8	180.5
野菜交付量 (g)						キャベツ 10							キャベツ 10							キャベツ 10			

第7表 1　1% Sodium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表　（g）

第V群																								
実験月日（1961）	15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
実験日数	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
飼育温度（°C）	21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
ラット体重（g） No. 1	198	198	198	208	212	211	210	203	215	213	224	223	221	236	231	228	225	235	234	232	237	235	240	240
No. 2	172	174	178	188	193	190	194	195	202	198	204	205	204	207	212	212	214	218	217	217	220	222	226	223
No. 3	194	195	195	203	209	207	204	209	208	209	218	217	216	216	217	215	214	221	221	229	222	225	228	225
No. 4	195	197	195	207	213	217	216	221	225	220	230	230	231	236	238	234	232	241	248	247	251	252	256	253
No. 5	194	195	195	205	208	211	214	215	219	217	228	227	224	228	236	233	230	239	241	242	241	241	245	241
No. 6	180	180	180	186	193	189	193	197	199	205	212	210	206	215	217	216	213	221	222	224	225	225	227	226
No. 7	188	194	193	202	210	206	208	217	216	217	228	229	228	234	238	236	235	243	254	249	254	257	258	258
No. 8	184	188	186	193	200	200	202	205	204	202	217	212	218	216	220	221	212	221	223	228	229	227	227	220
No. 9	175	177	178	183	188	189	188	191	190	196	199	200	208	204	205	204	198	205	206	210	212	211	214	213
No. 10	155	157	155	156	160	160	160	160	163	163	164	167	166	176	187	194	192	208	206	205	211	206	215	215
平均	183.5	185.5	185.3	193.1	198.6	198.0	198.9	201.3	204.1	204.0	212.4	212.0	212.2	216.8	220.1	219.3	216.5	225.2	227.2	228.3	230.2	230.1	233.6	231.4
野菜交付量（g）				キャベツ 10							キャベツ 10							キャベツ 10						
実験月日（1961）	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
実験日数	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	
飼育温度（°C）	22	22	22	22	22	22	22	23	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23	
ラット体重（g） No. 1	245	243	255	266	255	262	264	265	269	275	273	270	274	275	275	272	281	275	275	283	288	294	282	
No. 2	231	228	232	234	237	234	240	239	243	240	248	240	249	251	249	252	251	251	249	257	257	256	257	
No. 3	230	226	243	246	236	241	247	249	249	253	253	247	250	252	250	250	264	259	261	268	269	277	271	
No. 4	263	265	271	281	270	280	284	285	297	295	300	292	299	298	298	300	298	307	306	310	310	314	313	
No. 5	249	247	263	264	257	258	263	261	266	273	272	265	271	269	273	272	280	278	282	283	289	290	288	
No. 6	236	238	245	245	240	244	249	251	258	255	257	259	269	264	256	266	273	267	268	275	272	279	281	
No. 7	265	264	275	279	273	276	282	281	287	285	293	283	291	295	292	290	292	292	287	296	295	305	303	
No. 8	234	227	243	253	250	250	250	251	257	248	253	255	263	258	259	261	265	269	269	277	277	285	283	
No. 9	215	214	233	228	228	228	231	233	235	238	242	240	243	244	241	239	245	245	245	659	249	251	249	
No. 10	222	220	235	241	234	239	240	243	247	249	249	247	252	254	252	250	257	261	260	263	261	277	275	
平均	239.0	237.2	249.5	253.7	248.0	251.2	255.0	255.8	260.8	261.1	264.0	259.8	265.9	266.0	264.6	265.2	270.6	270.4	270.2	277.1	276.7	282.8	280.2	
野菜交付量（g）	キャベツ 10							キャベツ 10							キャベツ 10							キャベツ 10		

第7表 2　1% Sodium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表　（g）

第V群																								
実験月日（1962）	1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
実験日数	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	
飼育温度（°C）	22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22	
ラット体重（g） No. 1	294	300	297	303	302	306	309	312	310	306	301	304	308	313	306	309	314	310	312	314	317	313	315	
No. 2	256	261	260	261	262	262	267	263	265	266	263	261	262	268	262	265	268	266	274	273	278	273	277	
No. 3	272	277	278	280	277	281	278	280	282	285	278	277	278	295	289	289	296	290	292	298	283	296	303	
No. 4	318	315	313	319	314	322	321	319	321	317	315	318	319	334	323	331	334	330	333	335	333	332	336	
No. 5	295	293	289	292	295	300	301	300	306	300	296	299	296	304	297	303	305	302	303	306	306	300	302	
No. 6	281	286	288	282	285	287	293	289	291	293	290	300	297	304	296	300	300	296	298	298	304	306	302	
No. 7	299	308	313	308	306	312	314	312	310	313	307	308	305	312	309	304	315	313	313	322	322	316	324	
No. 8	281	283	288	286	296	289	293	293	297	294	286	300	298	300	287	300	304	300	297	301	298	299	304	
No. 9	246	249	253	250	256	256	256	260	262	262	264	264	270	272	267	271	280	275	278	278	274	273	277	
No. 10	277	284	284	285	283	288	290	287	291	287	289	287	289	291	284	290	291	295	293	296	296	294	299	
平均	281.9	285.6	286.3	286.6	287.6	270.3	292.2	291.5	293.5	292.3	288.1	291.8	292.2	299.1	297.0	296.2	300.7	297.7	299.3	302.1	301.1	300.2	303.9	
野菜交付量（g）						キャベツ 10							キャベツ 10							キャベツ 10				
実験月日（1962）	24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
実験日数	112	113	114	115	116	117	118	119	20	121	122	123	124	125	126	127	128	129	130	131	132	133	134	
飼育温度（°C）	22	21	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22	
ラット体重（g） No. 1	312	313	310	310	307	310	312	315	317	317	320	324	321	321	320	319	321	322	323	323	324	323	328	
No. 2	275	271	271	276	276	278	279	279	277	277	282	287	282	284	286	289	289	291	293	294	295	294	295	
No. 3	294	295	296	290	291	295	295	294	295	295	294	298	297	296	295	294	296	297	297	299	299	300	299	
No. 4	331	332	331	332	328	336	337	336	330	331	334	337	333	333	330	330	331	333	325	340	337	339	336	
No. 5	300	302	301	302	302	307	311	309	309	307	308	310	308	308	308	307	310	312	312	312	313	313	312	
No. 6	300	305	305	306	303	302	304	311	310	302	308	310	306	312	311	307	307	310	315	315	322	314	321	
No. 7	318	319	317	318	315	314	315	314	314	318	319	328	323	330	324	323	324	327	331	330	337	330	337	
No. 8	302	303	306	306	307	307	308	310	311	315	319	326	319	318	327	322	327	331	331	335	335	332	335	
No. 9	298	278	275	277	273	278	279	282	282	280	277	281	277	278	273	271	277	277	279	282	285	283	280	
No. 10	296	296	298	298	300	299	301	301	303	302	303	307	307	307	306	304	310	309	313	313	317	316	315	
平均	300.6	301.4	301.0	301.5	300.7	302.7	306.1	305.1	304.8	304.4	306.4	310.8	307.3	308.7	308.1	306.6	309.2	310.9	311.9	314.3	316.4	314.5	315.8	
野菜交付量（g）				キャベツ 10							キャベツ 10							キャベツ 10						

第7表 2　1% Sodium Propionate 含有飼料をラットに連続 180 日間経口交付せる場合の体重推移表 (g)

第V群 (2)

実験月日 (1962)	16/II	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
ラット体重 (g) No. 1	320	322	326	324	336	329	330	332	332	334	334	336	338	341	343	340	340	351	346	349	345	347	345
No. 2	296	291	296	295	300	299	302	299	300	303	298	302	306	306	308	305	305	310	307	309	308	308	307
No. 3	296	295	298	296	298	297	298	299	301	302	306	304	304	305	305	302	304	321	311	311	310	310	314
No. 4	339	337	338	337	342	341	342	342	342	342	347	347	346	352	352	346	352	340	358	356	351	353	356
No. 5	314	309	310	312	314	319	315	322	320	320	323	323	318	325	326	324	324	332	333	333	327	329	326
No. 6	316	316	328	316	317	318	320	323	327	330	329	330	330	335	335	328	334	343	336	335	335	334	337
No. 7	335	332	336	331	340	334	335	341	340	338	338	348	342	346	350	346	348	355	357	353	347	353	353
No. 8	333	330	332	325	334	335	332	337	336	338	342	347	346	342	343	342	346	353	349	351	349	349	345
No. 9	284	276	286	278	283	282	282	281	285	288	286	290	285	288	288	277	280	287	291	294	286	295	293
No. 10	317	315	315	317	319	320	321	324	328	334	330	332	334	335	333	333	336	349	341	344	337	342	343
平均	315.0	312.3	316.5	313.1	318.3	317.4	317.7	320.0	321.0	323.9	323.3	325.9	324.9	327.5	328.3	324.3	326.9	334.1	332.9	333.5	329.5	332.0	331.9
野菜交付量 (g)		キャベツ 10							キャベツ 10							キャベツ 10							キャベツ 10

実験月日 (1962)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
ラット体重 (g) No. 1	349	353	350	350	350	350	350	352	353	352	354	358	354	355	352	354	354	355	354	353	352	351	358
No. 2	310	309	313	315	309	311	310	312	318	314	314	320	316	312	313	320	314	312	314	314	315	314	317
No. 3	312	313	313	314	314	314	313	318	326	324	319	330	324	326	324	329	324	325	327	324	327	328	336
No. 4	356	356	358	357	358	357	360	358	360	364	365	358	362	362	360	364	365	365	366	362	364	363	366
No. 5	333	332	331	331	332	331	334	332	338	342	342	340	336	337	338	336	337	337	337	338	336	338	342
No. 6	340	349	345	348	345	344	344	342	351	357	353	353	347	348	348	357	350	350	352	354	353	349	360
No. 7	356	356	356	362	356	362	361	358	365	367	362	369	362	360	363	362	358	364	364	364	364	362	369
No. 8	351	355	356	351	353	351	357	356	362	365	359	360	359	363	358	365	368	372	371	370	373	371	376
No. 9	292	297	298	297	298	295	295	292	298	302	309	310	304	304	298	305	305	303	302	302	302	305	305
No. 10	343	353	349	346	346	343	345	344	350	347	345	347	346	345	346	352	348	347	346	346	347	348	356
平均	334.2	337.3	336.9	337.1	336.1	335.8	336.9	336.4	342.1	343.4	342.2	341.5	341.0	341.2	340.0	344.4	342.3	343.0	343.3	342.7	343.3	342.9	348.5
野菜交付量 (g)							キャベツ 10							キャベツ 10							キャベツ 10		

第8表 1　3% Sodium Propionate 含有飼料をラットに連続 180 日間経口交付せる場合の体重推移表 (g)

第VI群

実験月日 (1962)	30/IV	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
実験日数	1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
飼育温度 (°C)	22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21
ラット体重 (g) No. 1	88	87	98	104	114	交付開始	119	116	122	123	124	131	131	134	137	131	139	143	143	143	142	143	149	149
No. 2	79	77	89	89	102		107	108	119	118	123	120	121	127	129	128	131	132	136	137	138	141	144	148
No. 3	79	82	90	93	103		111	110	118	118	117	121	126	127	132	131	135	137	139	143	142	145	151	151
No. 4	77	76	85	91	94		98	99	106	109	111	109	116	116	117	120	121	123	122	118	115	126	121	122
No. 5	69	71	80	87	86		92	95	99	100	104	104	109	111	115	114	115	118	122	122	125	120	128	130
No. 6	71	70	81	85	87		95	95	103	106	111	108	110	115	118	120	121	127	130	130	132	134	141	137
No. 7	67	69	78	84	90		98	99	106	108	110	109	113	116	121	120	122	128	130	131	132	134	138	140
No. 8	68	64	75	82	86		95	96	104	106	109	113	115	117	123	122	127	133	135	137	139	137	141	140
No. 9	69	69	76	81	83		88	93	96	100	104	107	103	112	116	111	119	126	127	127	129	130	135	136
No. 10	63	62	71	79	84		84	90	98	96	99	99	97	103	104	106	108	109	113	114	117	117	121	121
平均	73.0	72.7	82.3	87.5	92.9		98.7	99.8	107.1	108.4	111.2	112.1	114.1	117.8	121.2	120.3	123.8	127.6	129.7	130.2	131.1	132.7	136.1	137.4
野菜交付量 (g)									キャベツ 10							キャベツ 10							キャベツ 10	

実験月日 (1962)	23	24	25	26	27	28	29	30	31	1/XI	2	3	4	5	6	7	8	9	10	11	12	13	14
実験日数	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
飼育温度 (°C)	21	21	21	22	23	23	23	23	23	23	22	23	23	23	21	22	22	22	22	22	22	21	21
ラット体重 (g) No. 1	151	150	155	154	155	158	163	170	166	171	170	178	175	178	179	183	188	189	188	193	191	197	199
No. 2	146	145	148	152	155	157	169	172	167	169	170	173	169	180	176	180	185	186	189	196	193	201	199
No. 3	154	150	155	157	158	162	170	173	170	173	174	176	178	183	180	184	189	189	190	190	189	194	195
No. 4	128	128	123	135	132	137	138	134	130	135	137	142	143	150	150	153	157	162	163	166	168	171	193
No. 5	131	130	135	136	136	142	145	154	150	153	155	160	159	159	167	170	173	174	176	181	180	190	190
No. 6	140	137	141	142	142	149	150	153	153	154	155	161	161	168	161	168	169	171	172	181	180	181	180
No. 7	140	139	143	143	143	148	151	155	152	154	156	160	156	167	162	168	170	171	172	180	179	182	185
No. 8	145	147	150	152	154	164	164	172	169	173	174	179	179	180	182	188	190	193	193	197	197	199	201
No. 9	139	138	140	142	143	150	154	160	158	159	160	166	166	174	172	178	177	180	180	183	182	190	189
No. 10	124	123	126	128	130	138	138	146	142	146	147	151	153	156	158	159	163	164	165	169	167	170	172
平均	139.8	138.7	141.6	144.1	144.8	150.5	154.2	158.9	155.7	158.7	159.8	164.6	163.9	169.5	168.7	173.1	176.1	177.9	178.8	183.6	182.6	187.5	188.3
野菜交付量 (g)						キャベツ 10							キャベツ 10							キャベツ 10			

第8表 1　3% Sodium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表（g）

実験月日（1962）	15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
実験日数	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
飼育温度（°C）	21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	21	22	22
ラット体重（g） No. 1	200	201	201	208	216	214	214	222	221	226	233	235	234	242	245	244	241	250	253	253	254	260	264	262
No. 2	202	201	202	211	217	218	215	220	221	224	231	233	228	234	237	233	232	240	241	243	242	246	248	250
No. 3	198	197	196	206	213	214	214	218	220	222	230	230	228	229	235	234	230	242	213	242	246	247	249	249
No. 4	175	173	177	184	190	187	192	193	225	215	205	205	200	206	208	207	202	213	216	217	219	218	220	220
No. 5	192	194	195	204	212	207	206	205	201	224	235	232	228	236	233	244	232	245	256	245	253	256	259	254
No. 6	183	183	182	187	194	195	196	203	206	205	212	212	207	211	215	216	209	219	225	225	227	225	227	231
No. 7	189	191	183	200	199	195	198	202	225	205	214	210	214	214	219	218	215	220	224	229	233	230	231	231
No. 8	204	206	208	216	223	221	221	226	218	224	237	238	233	237	243	238	238	249	253	254	258	258	262	259
No. 9	190	194	193	201	212	212	216	215	196	220	228	234	230	231	238	234	234	238	244	240	244	245	244	246
No. 10	176	179	177	185	190	192	190	194	196	199	205	210	212	212	215	211	209	220	220	223	221	227	226	227
平均	190.9	191.9	191.4	200.2	206.6	205.5	206.2	209.8	212.9	214.4	223.0	223.9	222.4	225.2	228.8	227.9	224.2	233.6	237.0	237.1	239.7	242.2	243.0	242.9
野菜交付量（g）				キャベツ 10							キャベツ 10							キャベツ 10						

実験月日（1962）	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
実験日数	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88
飼育温度（°C）	22	22	22	22	22	22	22	23	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23
ラット体重（g） No. 1	269	271	280	284	285	285	288	291	290	294	298	291	296	294	290	290	294	292	291	296	290	289	289
No. 2	257	255	265	275	269	272	278	277	282	279	283	278	286	283	280	288	290	288	291	295	280	299	290
No. 3	256	254	266	273	264	272	278	279	278	276	285	282	289	282	285	282	295	295	294	300	295	305	299
No. 4	226	222	237	240	238	244	249	249	247	243	249	248	251	248	251	252	251	252	253	257	255	258	253
No. 5	261	260	270	279	276	277	282	285	289	287	291	290	295	293	291	290	293	292	296	299	294	303	302
No. 6	243	237	253	254	244	251	259	257	267	268	273	266	271	273	278	278	283	284	272	291	285	297	298
No. 7	237	235	253	255	245	250	255	254	263	259	264	267	272	268	270	272	274	278	276	282	278	299	296
No. 8	271	269	284	285	277	286	289	289	297	295	299	301	304	301	297	297	301	302	295	304	300	320	327
No. 9	250	248	253	257	256	257	260	264	265	264	267	264	263	268	266	270	271	272	271	275	274	280	277
No. 10	239	235	249	258	246	253	260	260	267	268	268	266	276	274	275	275	285	293	290	294	290	301	300
平均	250.9	248.6	261.0	266.0	260.0	264.7	269.7	270.5	274.5	275.4	277.7	275.3	280.3	278.4	278.3	279.4	283.7	284.8	292.9	289.3	284.8	295.1	293.1
野菜交付量（g）	キャベツ 10							キャベツ 10							キャベツ 10							キャベツ 10	

第VI群

第8表 2　3% Sodium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表（g）

実験月日（1962）	1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
実験日数	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
飼育温度（°C）	22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22
ラット体重（g） No. 1	290	294	293	286	289	294	296	293	299	298	287	295	291	292	291	300	299	298	299	302	303	305	311
No. 2	292	295	299	292	296	297	298	296	301	295	298	303	298	308	303	303	305	306	302	304	307	305	310
No. 3	301	303	307	302	301	304	306	305	314	310	303	306	308	317	307	314	315	312	311	314	314	311	315
No. 4	258	257	259	257	257	265	266	259	273	272	266	271	277	284	277	275	283	277	283	288	288	279	289
No. 5	302	300	301	300	302	297	300	289	306	303	300	303	301	309	303	308	315	311	307	312	308	309	315
No. 6	297	300	296	300	301	303	305	306	308	314	310	308	303	314	307	312	318	320	322	324	328	323	334
No. 7	300	314	283	305	305	289	304	311	306	306	298	304	302	313	303	312	313	312	315	315	315	321	319
No. 8	336	324	330	344	341	335	338	349	344	340	337	345	340	355	343	350	353	352	346	354	352	355	359
No. 9	286	280	278	280	281	279	283	277	284	283	280	286	284	288	288	290	287	290	292	297	301	298	303
No. 10	304	309	300	307	310	313	317	311	316	319	312	320	319	330	313	316	331	331	333	338	336	339	344
平均	296.6	297.6	294.6	297.3	298.3	297.6	301.5	299.6	305.1	304.0	299.1	304.1	302.3	311.0	303.0	308.0	311.9	310.9	311.0	314.8	315.2	314.5	319.9
野菜交付量（g）						キャベツ 10							キャベツ 10							キャベツ 10			

実験月日（1962）	24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15
実験日数	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	11	130	131	132	133	134
飼育温度（°C）	22	12	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22
ラット体重（g） No. 1	308	309	309	310	306	313	310	312	310	311	317	318	313	316	314	314	316	315	321	322	323	324	326
No. 2	301	302	305	303	302	303	308	304	304	300	304	308	305	306	303	306	307	308	311	310	311	310	311
No. 3	313	318	316	313	317	313	315	316	316	316	318	327	321	322	319	321	322	320	324	324	333	327	331
No. 4	288	286	287	280	291	292	295	299	298	298	298	303	296	301	301	302	298	301	304	307	308	307	299
No. 5	306	308	308	306	307	306	305	306	307	307	308	309	307	311	309	308	310	310	312	310	314	314	315
No. 6	321	323	320	322	319	330	330	329	332	329	331	341	336	335	330	332	335	340	341	343	342	343	344
No. 7	317	317	322	327	327	326	325	324	324	333	329	332	326	333	328	335	332	332	335	337	348	338	340
No. 8	354	354	355	355	355	353	355	357	363	363	364	370	361	355	362	367	368	363	368	366	370	368	371
No. 9	298	298	298	297	298	301	304	300	300	302	303	304	301	301	300	300	302	302	303	307	307	304	305
No. 10	337	338	338	337	339	337	335	338	341	338	337	343	341	342	342	342	342	347	349	351	352	349	353
平均	314.3	315.3	305.8	315.0	316.1	317.4	318.2	318.5	319.5	319.4	320.9	325.6	320.7	322.8	320.8	322.7	323.2	323.8	326.8	327.7	331.3	328.4	329.5
野菜交付量（g）				キャベツ 10							キャベツ 10							キャベツ 10					

第VI群

第8表 2　3% Sodium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表（g）

第VI群

実験月日 (1962)	16/II	17	18	19	20	21	22	23	24	25	26	27	28	1/III	2	3	4	5	6	7	8	9	10
実験日数	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157
飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	22	22	22	22	22	22	22	22
ラット体重 (g) No. 1	323	320	324	325	328	330	330	334	331	336	341	340	340	345	347	345	347	357	353	353	347	352	351
No. 2	311	305	312	312	315	314	314	320	320	316	325	326	325	334	333	326	332	339	337	337	328	332	336
No. 3	335	327	334	331	333	336	337	312	340	332	342	343	341	344	348	342	346	357	351	353	347	350	346
No. 4	306	303	308	309	318	315	317	320	317	316	322	326	324	327	330	327	330	333	331	334	335	335	338
No. 5	313	310	314	313	316	315	316	323	320	318	322	324	323	325	329	326	332	338	333	335	333	331	330
No. 6	346	342	346	313	356	354	353	357	356	356	362	361	366	364	367	363	365	364	372	373	367	371	378
No. 7	338	335	341	334	343	347	340	345	344	344	345	345	345	350	350	351	350	362	368	363	359	356	357
No. 8	368	361	370	365	373	372	373	375	373	374	377	377	381	386	380	380	380	391	384	388	383	392	384
No. 9	304	300	311	308	315	311	308	312	310	304	316	323	322	327	319	318	325	325	313	335	319	329	322
No. 10	351	348	350	354	359	355	356	353	351	346	354	356	356	356	358	355	347	363	359	356	353	357	351
平均	329.5	325.4	331.0	329.4	335.6	334.9	334.4	338.1	336.5	334.2	340.6	342.1	342.3	345.8	346.1	343.3	345.4	352.9	350.1	352.7	347.1	350.5	349.3
野菜交付量 (g)		キャベツ 10							キャベツ 10							キャベツ 10							キャベツ 10

第VII群

実験月日 (1962)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/IV	2
実験日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
飼育温度 (°C)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	23	23	23	22	22	22	22	22	22
ラット体重 (g) No. 1	356	350	350	350	346	344	342	342	326	332	329	325	322	317	311	309	303	312	313	316	317	320	326
No. 2	337	341	340	342	337	339	340	340	341	349	345	348	345	346	345	346	346	346	346	343	345	341	348
No. 3	352	350	353	352	349	352	353	348	356	358	355	359	360	355	352	350	350	353	356	352	355	352	358
No. 4	336	336	341	340	340	338	338	340	339	343	342	343	347	346	346	344	346	348	348	346	341	347	350
No. 5	333	329	336	333	335	334	332	334	341	343	340	340	339	338	339	345	342	345	340	342	341	340	343
No. 6	370	370	369	378	377	383	379	380	383	385	380	386	384	384	382	389	387	388	384	389	387	383	400
No. 7	354	356	354	361	362	355	357	361	359	360	362	366	364	372	366	372	367	368	364	362	364	362	367
No. 8	384	385	383	386	384	387	384	390	396	396	390	389	389	388	386	393	391	389	386	387	388	387	403
No. 9	324	329	329	328	324	326	323	330	333	334	332	329	333	330	328	336	331	332	335	332	332	330	337
No. 10	354	352	353	350	351	348	353	350	356	359	353	355	350	350	352	362	359	356	360	357	357	353	359
平均	350.0	349.2	350.8	352.0	350.5	350.6	350.1	351.4	353.0	353.2	352.8	354.0	353.3	352.6	350.7	354.6	352.2	353.7	353.2	352.6	352.7	351.5	359.1
野菜交付量 (g)							キャベツ 10							キャベツ 10							キャベツ 10		

第9表 1　6% Sodium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表（g）

第VII群

実験月日 (1962)	30/IX	1/X	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
実験日数	1	2	3	4	5		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
飼育温度 (°C)	22	23	23	23	23		23	23	23	22	22	22	22	23	23	22	22	23	23	23	23	22	21	21
ラット体重 (g) No. 1	89	85	97	105	106	交付開始	103	101	112	112	114	113	117	121	125	124	123	128	132	133	132	132	137	136
No. 2	85	81	93	96	103		106	103	113	117	121	121	122	124	131	130	130	134	136	139	139	139	141	141
No. 3	80	79	87	93	96		92	96	104	106	108	110	110	115	121	127	120	123	123	125	125	126	129	126
No. 4	74	73	83	91	92		94	94	105	108	113	114	117	120	125	127	128	127	128	130	132	129	136	134
No. 5	71	69	80	87	91		94	95	100	102	109	114	118	118	126	127	128	134	131	128	122	124	119	122
No. 6	72	70	79	85	91		93	94	101	104	108	112	117	116	120	120	125	125	132	130	128	130	133	134
No. 7	72	69	79	84	90		91	91	97	101	108	105	107	112	114	119	121	125	126	126	128	128	133	132
No. 8	80	78	85	83	94		98	101	96	105	110	116	118	114	120	120	122	122	126	127	127	129	130	130
No. 9	68	69	77	81	88		81	90	95	100	104	100	103	106	110	109	112	111	118	119	120	121	126	128
No. 10	67	65	76	79	83		87	90	96	101	105	106	107	104	115	114	120	114	117	116	116	117	124	130
平均	75.8	73.8	83.6	88.4	93.4		93.9	95.5	101.9	105.6	110.0	111.1	113.6	115.0	120.7	121.7	122.9	124.3	126.9	127.3	126.9	127.5	130.8	131.3
野菜交付量 (g)									キャベツ 10							キャベツ 10							キャベツ 10	

第VII群

実験月日 (1962)	23	24	25	26	27	28	29	30	31	1/XI	2	3	4	5	6	7	8	9	10	11	12	13	14
実験日数	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
飼育温度 (°C)	21	21	21	22	23	23	23	23	23	23	22	22	22	22	22	22	22	22	22	22	22	22	22
ラット体重 (g) No. 1	139	141	145	146	146	154	155	159	160	165	165	168	169	172	175	178	182	182	180	186	184	185	187
No. 2	142	140	146	145	145	153	158	159	157	157	160	163	165	170	170	172	175	179	179	184	185	186	184
No. 3	129	130	133	132	133	139	143	152	146	148	150	154	152	155	155	161	163	160	160	169	169	173	172
No. 4	139	138	138	139	139	145	147	154	152	155	155	159	157	164	163	167	172	174	174	179	178	178	181
No. 5	129	128	128	129	129	134	138	143	143	144	147	150	154	155	156	156	159	158	158	162	161	161	166
No. 6	139	140	142	145	145	152	152	161	159	160	155	166	161	167	166	170	173	174	173	175	176	177	178
No. 7	132	135	137	137	139	143	145	145	153	148	151	154	156	157	154	158	163	163	161	170	169	170	170
No. 8	133	133	137	137	137	141	142	149	146	146	148	152	152	155	153	158	163	161	163	170	170	169	170
No. 9	129	130	131	131	133	140	144	155	150	152	152	156	152	166	151	165	170	171	172	176	176	178	180
No. 10	125	125	128	129	129	138	138	146	148	142	150	151	148	154	152	156	159	158	158	160	161	162	163
平均	133.6	134.0	136.5	137.0	137.5	143.8	146.2	152.3	151.4	152.0	153.3	157.3	156.6	161.5	159.5	161.1	167.9	168.3	168.8	173.1	172.9	174.4	175.1
野菜交付量 (g)						キャベツ 10							キャベツ 10							キャベツ 10			

第9表 1 6% Sodium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表 (g)

実験月日 (1962)		15/XI	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1/XII	2	3	4	5	6	7	8
実験日数		42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
飼育温度 (°C)		21	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	21	22	22
ラット体重 (g)	No. 1	190	193	190	201	210	205	209	211	215	214	228	226	223	229	233	230	225	234	241	243	241	242	241	241
	No. 2	192	193	190	201	214	208	209	211	213	213	228	226	222	226	229	226	225	237	236	237	238	242	241	243
	No. 3	175	178	178	184	196	192	195	500	201	201	213	215	212	216	222	218	215	222	227	230	237	233	236	233
	No. 4	185	187	185	196	200	200	205	210	212	217	227	225	221	224	229	227	225	235	237	240	241	241	245	242
	No. 5	170	173	175	183	189	189	194	194	197	198	208	210	208	212	216	217	216	222	223	225	228	230	229	231
	No. 6	182	183	183	190	200	194	196	211	215	213	216	219	212	222	225	222	227	225	231	229	231	233	230	237
	No. 7	179	179	183	189	191	191	198	200	203	202	205	203	203	210	217	216	213	215	219	220	222	225	225	225
	No. 8	174	178	178	183	195	190	193	205	303	203	212	215	209	215	220	219	219	228	229	233	234	238	240	237
	No. 9	187	190	188	198	207	206	208	212	213	217	227	230	225	226	231	236	234	213	241	251	256	257	256	259
	No. 10	168	169	171	176	178	180	185	786	191	194	204	205	201	210	212	214	211	215	218	218	222	218	225	221
	平均	180.2	182.3	182.1	190.1	198.0	195.5	199.2	204.0	206.3	207.2	216.8	218.4	213.0	219.0	223.4	222.5	221.0	227.6	230.5	232.6	235.0	235.9	237.0	236.9
野菜交付量 (g)					キャベツ 10							キャベツ 10							キャベツ 10						

実験月日 (1962)		9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
実験日数		66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88
飼育温度 (°C)		22	22	22	22	22	22	22	22	21	21	22	22	22	22	22	22	22	21	21	21	22	23	23
ラット体重 (g)	No. 1	219	249	225	259	251	257	264	259	265	265	269	264	267	268	268	270	275	274	271	285	283	299	295
	No. 2	249	249	265	273	264	270	275	273	277	279	281	287	294	295	293	295	292	298	302	298	302	308	303
	No. 3	241	237	248	256	256	254	257	257	257	263	265	266	264	267	269	271	273	276	278	281	277	283	277
	No. 4	251	248	263	264	257	262	264	267	271	274	277	275	277	281	280	282	283	288	294	298	300	316	303
	No. 5	231	235	244	248	243	243	245	246	254	255	262	255	261	262	259	260	261	258	263	275	270	276	273
	No. 6	237	239	244	248	248	247	255	255	255	254	258	256	260	263	262	262	268	268	269	271	268	277	263
	No. 7	228	230	231	238	235	238	243	239	240	245	245	245	252	251	253	254	261	257	258	261	260	262	258
	No. 8	215	212	258	260	253	260	263	263	268	273	273	274	278	278	280	279	283	280	282	290	300	308	308
	No. 9	261	260	270	247	267	271	275	273	285	277	277	284	286	288	285	288	287	292	294	304	301	312	306
	No. 10	225	225	238	232	228	231	236	231	231	233	233	237	238	238	238	238	241	241	243	248	214	247	247
	平均	241.7	241.4	251.6	255.2	250.2	253.3	257.7	256.3	253.3	261.8	265.9	264.3	267.7	269.1	268.7	269.9	272.4	273.2	274.4	281.1	280.8	288.8	283.3
野菜交付量 (g)		キャベツ 10							キャベツ 10							キャベツ 10								キャベツ 10

第9表 2 6% Sodium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重推移表 (g)

実験月日 (1962)		1/I	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
実験日数		89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
飼育温度 (°C)		22	22	23	22	22	21	21	22	21	22	21	21	21	21	22	22	23	22	21	21	22	21	22
ラット体重 (g)	No. 1	293	295	294	288	293	291	296	295	294	293	290	291	288	295	284	299	295	293	290	296	295	293	300
	No. 2	309	310	317	314	318	315	317	315	314	309	312	316	313	315	305	317	321	316	314	319	320	323	323
	No. 3	275	275	281	274	275	278	288	275	283	282	284	289	283	285	288	281	293	290	293	293	300	293	300
	No. 4	315	314	319	317	318	323	317	322	317	312	310	315	315	317	309	319	321	321	320	323	324	316	324
	No. 5	275	273	279	275	278	279	281	279	282	278	280	280	286	288	277	290	296	291	290	293	294	290	297
	No. 6	265	273	274	269	270	273	277	267	277	280	277	278	278	289	274	283	287	289	290	292	290	290	302
	No. 7	252	263	263	263	263	265	265	270	273	276	275	279	282	285	279	284	281	286	293	290	290	292	294
	No. 8	309	311	314	317	318	319	317	315	314	313	313	316	317	319	309	322	323	317	321	322	316	319	326
	No. 9	311	312	311	309	319	308	316	315	313	310	314	320	317	319	316	318	321	319	322	325	327	323	328
	No. 10	243	244	242	242	243	241	243	244	247	242	246	250	250	254	250	252	255	255	258	262	264	260	266
	平均	281.7	287.0	289.4	286.8	289.5	290.2	291.7	289.7	291.4	289.5	270.2	293.4	292.9	296.6	289.1	296.5	299.3	297.7	299.1	301.5	302.0	299.9	306.0
野菜交付量 (g)							キャベツ 10							キャベツ 10							キャベツ 10			

実験月日 (1962)		24	25	26	27	28	29	30	31	1/II	2	3	4	5	6	7	8	9	10	11	12	13	14	15
実験日数		112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
飼育温度 (°C)		22	21	22	21	22	22	22	22	22	23	22	22	22	22	22	22	22	22	22	22	22	22	22
ラット体重 (g)	No. 1	292	294	295	290	289	294	290	287	287	285	288	294	290	287	290	289	293	292	297	294	294	294	293
	No. 2	320	322	318	315	315	318	312	315	311	313	315	321	316	319	314	314	315	314	316	318	321	313	318
	No. 3	290	287	292	285	289	292	287	292	284	285	291	299	293	292	291	292	294	293	302	299	298	297	300
	No. 4	319	319	319	311	312	310	308	309	306	306	308	308	304	305	304	303	303	302	303	300	301	301	300
	No. 5	293	294	291	291	291	294	293	294	291	295	298	299	294	291	293	293	294	294	297	296	296	295	299
	No. 6	292	295	293	293	293	299	294	296	298	293	291	303	294	296	292	290	294	294	296	299	298	294	298
	No. 7	291	292	297	294	291	296	293	297	297	295	299	304	290	297	294	295	298	300	302	298	303	305	306
	No. 8	322	318	316	314	317	317	317	317	316	315	318	322	315	317	315	315	320	317	317	322	322	316	319
	No. 9	322	330	324	325	325	323	320	322	318	318	322	323	318	323	320	320	321	324	321	329	327	322	326
	No. 10	254	255	250	255	257	257	259	261	258	257	260	258	261	261	263	262	264	266	267	266	271	268	271
	平均	299.5	300.4	299.9	297.3	297.9	300.0	297.3	299.0	296.6	296.2	299.0	303.1	297.5	299.1	297.6	297.3	299.6	299.6	302.1	302.1	313.1	300.5	303.0
野菜交付量 (g)					キャベツ 10							キャベツ 10							キャベツ 10					

第9表 2　6% Sodium Propionate 含有飼料をラットに連続180日間経口投与する場合の体重推移表 (g)

群	実験日数 (1962)	10/11	17	18	19	20	21	22	23	24	25	26	27	28	11/IV	2	3	4	5	6	7	8	9	10
	飼育日数	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
無処置対照	No. 1	291	288	298	295	300	298	297	302	300	298	307	302	302	303	304	301	304	309	303	311	303	310	306
	No. 2	315	312	316	311	316	311	315	317	314	311	321	318	318	322	323	319	319	328	328	328	322	322	327
	No. 3	300	295	303	301	304	300	305	308	311	312	317	320	314	318	318	315	316	327	329	326	317	321	323
	No. 4	301	296	302	296	302	300	301	307	308	306	311	310	312	309	311	309	312	323	323	323	320	320	321
	No. 5	295	291	299	294	301	306	304	308	308	307	307	311	312	314	315	309	308	320	319	318	315	319	320
	No. 6	296	295	295	278	293	290	291	295	293	300	302	302	303	307	307	306	310	319	318	317	313	319	317
	No. 7	305	303	305	284	295	294	298	299	302	304	303	303	304	308	311	304	310	317	315	315	307	313	315
	No. 8	317	314	316	298	309	309	311	316	314	312	312	315	314	319	315	317	317	330	329	329	321	328	327
	No. 9	322	320	324	305	314	317	319	318	318	315	317	325	321	330	327	324	325	334	333	335	331	335	336
	No. 10	271	267	266	250	264	260	261	263	259	257	267	273	268	274	278	275	279	280	284	287	283	281	284
	平均	301.6	298.1	302.5	291.2	299.8	298.5	300.2	303.6	302.6	301.2	306.0	307.9	306.8	310.8	316.1	307.7	310.0	318.7	318.1	318.9	313.2	317.6	317.6
体重 (g)																								
群	実験日数 (1962)	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	11/IV	2	3	4	5
	飼育日数	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
	飼育温度 (℃)	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
C.P. 1%	No. 1	309	315	318	317	313	317	315	317	319	320	320	316	316	315	313	314	314	315	313	314	315	312	320
	No. 2	322	331	331	332	329	331	331	330	338	337	336	336	333	331	330	331	332	332	331	332	332	328	337
	No. 3	323	334	325	324	321	325	323	328	332	338	328	323	323	325	324	326	328	328	323	324	323	320	329
	No. 4	321	323	321	324	323	324	322	325	328	328	329	324	324	324	320	322	324	324	324	324	317	319	322
	No. 5	318	322	327	326	322	322	321	323	328	335	335	327	323	324	322	323	324	322	322	325	324	323	330
	No. 6	325	329	334	333	330	334	331	326	335	339	331	337	334	338	336	334	338	336	336	335	334	333	340
	No. 7	316	317	317	318	317	317	321	316	315	322	318	320	320	321	320	324	321	322	325	321	321	323	328
	No. 8	327	325	327	331	334	331	331	332	336	336	332	330	330	334	330	336	335	334	335	331	333	332	337
	No. 9	335	314	335	335	335	335	339	338	343	345	340	339	340	334	333	342	335	334	333	335	331	337	346
	No. 10	283	285	294	296	291	296	297	293	298	295	298	293	299	296	293	294	299	299	298	299	298	297	300
	平均	317.0	322.8	322.9	323.6	321.3	323.4	323.3	322.8	327.0	329.5	326.4	324.6	324.5	324.7	322.2	324.2	324.9	324.2	324.0	324.8	323.6	323.8	328.9
体重 (g)																								

Table 10

第10表　Caicium Propionate 及び Sodium Propionate を連続180日間投与する場合の各群ラットの第181日の血球数，血色素量，色素指数及び白血球百分比

ラットの群別	番号	性別	体重 (g)	血球数 赤血球数	血球数 白血球数	血色素 (%)	色素指数	白血球百分比 B	E	N St	N S	L	Mo	Pl
I 無処置対照	No. 1	♂	304	725	8,900	85	.59	0	0	1	57	40	2	0
	No. 2	〃	378	835	9,300	96	.58	0	0	11	27	59	3	0
	No. 3	〃	332	795	7,500	85	.53	0	0	7	35	55	3	0
	No. 4	〃	286	820	8,400	88	.54	0	1	3	13	82	1	0
	No. 5	〃	300	820	9,700	83	.51	0	1	0	21	76	2	0
	No. 6	〃	324	735	7,500	89	.60	0	2	7	27	63	1	0
	No. 7	〃	389	790	8,300	90	.60	0	2	8	33	51	6	0
	No. 8	〃	312	790	6,900	83	.53	0	3	10	35	49	3	0
	No. 9	〃	339	835	6,900	85	.52	0	1	9	34	56	0	0
	No. 10	〃	295	845	8,100	92	.54	0	1	10	28	60	1	0
	(7) 平均		325.9	796	8,150	88	.55	0	1.1	6.6	31.0	59.1	2.2	0
II C.P. 1%	No. 1	♂	315	775	12,300	79	.52	1	1	11	4	73	5	0
	No. 2	〃	340	785	4,400	90	.59	0	4	20	12	58	6	0
	No. 3	〃	311	790	6,700	85	.61	0	1	22	9	61	7	0
	No. 4	〃	336	870	8,100	86	.44	0	0	13	5	72	10	0
	No. 5	〃	341	775	7,300	87	.59	0	1	11	21	67	0	0
	No. 6	〃	328	785	6,800	85	.54	0	1	21	13	60	2	0
	No. 7	〃	364	770	7,600	89	.66	1	1	16	12	63	7	0
	No. 8	〃	353	785	7,800	78	.55	0	1	18	7	68	6	0
	No. 9	〃	371	815	6,600	82	.57	0	0	21	25	50	4	0
	No. 10	〃	329	765	8,400	89	.58	0	0	17	8	70	5	0
	(7) 平均		338.8	786	7,600	85	.57	0.2	1.0	17.3	11.6	64.7	5.2	0
III C.P. 3%	No. 1	♂	323	775	7,900	92	.63	0	1	12	6	70	11	0
	No. 2	〃	358	775	6,700	93	.65	0	0	10	7	83	0	0
	No. 3	〃	353	795	8,300	93	.50	0	0	21	6	64	9	0
	No. 4	〃	379	795	6,900	88	.56	0	0	13	5	78	4	0
	No. 5	〃	320	835	9,400	100	.52	0	0	14	8	73	5	0
	No. 6	〃	376	885	9,000	99	.85	0	0	14	7	76	3	0
	No. 7	〃	338	875	9,300	96	.57	0	3	14	5	73	5	0
	No. 8	〃	332	925	12,800	100	.54	0	0	8	6	76	10	0
	No. 9	〃	337	875	7,700	92	.50	0	3	11	3	77	6	0
	No. 10	〃	306	855	7,900	94	.58	0	4	9	2	77	8	0
	(10) 平均		342.2	817	8,890	95	.59	0	1.1	12.6	5.5	74.7	6.1	0
IV C.P. 6%	No. 1	♂	365	875	9,200	94	.56	0	3	11	9	72	5	0
	No. 2	〃	354	835	10,200	82	.67	0	0	19	4	73	4	0
	No. 3	〃	336	775	6,300	86	.79	1	2	12	3	79	3	0
	No. 4	〃	303	765	5,800	83	.57	1	5	13	7	71	3	0
	No. 5	〃	334	775	7,500	82	.62	0	1	14	12	66	7	0
	No. 6	〃	332	825	8,900	93	.75	0	2	5	5	83	5	0
	No. 7	〃	371	815	11,000	92	.63	0	1	14	4	77	4	0
	No. 8	〃	354	785	10,300	86	.61	0	1	23	9	56	11	0
	No. 9	〃	330	785	7,200	87	.64	2	0	11	12	72	3	0
	No. 10	〃	299	785	6,900	93	.70	0	0	14	6	80	0	0
	(8) 平均		337.8	795	8,330	88	.65	0.4	1.5	13.6	7.1	72.9	4.5	0
V S.P. 1%	No. 1	♂	358	825	7,100	91	.57	0	2	20	5	70	3	0
	No. 2	〃	317	775	6,500	95	.56	0	1	17	1	73	8	0
	No. 3	〃	336	825	7,600	92	.60	0	2	22	4	62	10	0
	No. 4	〃	366	775	5,600	87	.69	1	2	25	11	54	6	0
	No. 5	〃	342	775	5,800	80	.52	1	0	20	9	65	5	0
	No. 6	〃	360	785	6,400	81	.50	0	11	17	1	66	5	0
	No. 7	〃	369	785	5,800	86	.61	0	1	11	9	78	1	0
	No. 8	〃	376	795	5,000	80	.65	0	2	20	8	70	1	0
	No. 9	〃	305	775	6,900	82	.43	0	1	20	1	57	21	0
	No. 10	〃	356	795	5,400	87	.70	0	0	13	3	76	8	0
	(6) 平均		348.5	781	6,210	86	.58	0.2	2.1	18.6	5.2	67.1	6.8	0

ラットの群別	番号	性別	体重 (g)	血球数 赤血球数	血球数 白血球数	血色素 %	血色素 指数	白血球百分比 B	E	N St	N S	L	Mo	Pl
VI S.P. 3%	No. 1	♂	326	605	9,100	82	.72	0	0	13	6	68	13	0
	No. 2	〃	348	635	8,500	81	.64	0	0	23	13	63	1	0
	No. 3	〃	358	630	7,200	85	.68	0	1	28	4	60	7	0
	No. 4	〃	350	580	8,600	84	.72	0	2	13	3	76	6	0
	No. 5	〃	313	650	7,300	86	.66	0	0	13	8	77	2	0
	No. 6	〃	400	845	8,700	97	.57	0	0	20	7	68	5	0
	No. 7	〃	367	845	10,300	99	.58	0	0	26	12	58	4	0
	No. 8	〃	403	680	7,200	92	.68	0	0	10	9	73	8	0
	No. 9	〃	337	820	7,800	97	.57	0	0	20	8	71	1	0
	No. 10	〃	359	780	9,800	97	.62	0	6	23	16	54	1	0
	平均		359.1	707	8,450	90	.64	0	0.9	18.9	8.6	66.8	4.8	0
VII S.P. 6%	No. 1	♂	320	620	8,700	91	.73	0	6	10	8	73	3	0
	No. 2	〃	337	690	7,800	94	.68	0	1	2	34	56	7	0
	No. 3	〃	329	740	9,900	95	.64	0	2	7	18	67	6	0
	No. 4	〃	322	785	10,400	100	.64	0	4	17	31	47	1	0
	No. 5	〃	330	690	7,600	98	.71	0	2	7	12	72	7	0
	No. 6	〃	340	685	7,600	93	.68	1	5	12	17	58	7	0
	No. 7	〃	328	655	7,200	89	.68	0	2	9	13	70	6	0
	No. 8	〃	337	730	5,600	90	.62	0	0	14	16	65	5	0
	No. 9	〃	346	855	6,100	96	.56	0	1	6	5	84	4	0
	No. 10	〃	300	760	5,200	95	.62	0	5	18	9	62	6	0
	平均		328.9	721	7,610	94	.66	0.1	2.8	10.2	16.3	65.4	5.2	0

併し乍ら C.P. と S.P. の相互間では C.P. の方の毒性が強いようである.

2. C.P. 及び S.P. の慢性毒性実験

Wister 系雄性ラットを用い, 6カ月 (180 日間) にわたり慢性毒性実験を行つた.

ラットに被検物質を経口的に交付して, その毒性を検索する場合, 殊に毒性が僅少と予測される物質の検索は発育曲線を中軸として行い, 更に一般状態を精細に観察することが緊要である. 最も適切なる発育曲線を得るためには, 実験動物は系統が統一され, 且つ一定度に成熟に近ずいた健全なるものが望ましい. 従つて余等は 80 g 前後の Wister 系雄性ラットを必要動物数の約倍数恒温実験室内で一週間以上飼育観察し, 一般状態の佳良なもののみ選別して本実験に供した.

飼育方法は恒温室内で小山式恒温飼育箱を使用し, 飼育温度を常に 22°C±1°C に保ち 1ヶの Cage 中に 1匹を収容して 10 匹1群とした. 飼料はオリエンタル粉末飼料 N.M.F. を使用し, 体重 100 g に対し 15 g を基準として交付した. 尚, 毎日総合ビタミン剤水溶液を飲料水中に 1 gtt/100 ml の割合に添加し, また7日に一度新鮮な野菜の交付を行つた.

被検物質である C.P. 及び S.P. は 1%, 3% 及び 6% 含有飼料を用いたが, その交付基準は次のごとき想定のもとに算出した. 即ち, 日本イースト株式会社の調査及び研究によりプロピオン酸塩は食パン製品 100 g に対して 0.09 乃至 0.18 g 添加される. 従つて本教室においてはその最大限を 0.2 g として毒性の検索を行つた. 即ち, 人が一日に摂取するパンの量を 150 g とすれば体重 60 kg の人間が 1 日に摂取するプロピオン酸塩の量は 0.3 g/60 kg (5 mg/kg) となる. 余等はこの量を以て本実験の基準量とした. 而して, 1%, 3% 及び 6% 各含有飼料交付群のラットが摂取する量的関係は, ラット 1 匹につき基準量の平均 300 倍, 900 倍及び 1,800 倍である. 即ち, 次の如く 1 日交付量に依り 7 群に分けて実験を行つた.

(1) 第1群：無処置処対照
(2) 第2群：1% C.P. 含有飼料交付
(3) 第3群：3% C.P. 含有飼料交付
(4) 第4群：6% C.P. 含有飼料交付
(5) 第5群：1% S.P. 含有飼料交付
(6) 第6群：3% S.P. 含有飼料交付
(7) 第7群：6% S.P. 含有飼料交付

かくして毎日一定時刻に 0.5 g の感度を有する自動天秤を用いて体重の測定を行い, その際飼料及び水分の摂取状態も観察した.

またラットの運動状態, 食欲, 毛並みの状態, 皮膚病発生の有無, 鼻出血の有無, 眼変化その他の一般状態も観察した.

180 日間連日試料交付後直ちに採血して血液学的検索を行い, 然る後失血致死せしめて諸臓器の重量及び容積を測定し, 対照のそれと比較検索を行つた. 更に各臓器をホルマリンに固定して病理組織学的検索を行つた.

1) C.P. 及び S.P. の各%含有飼料連続 180 日間交付における成長発育実験

(1) 第1群：無処置飼育による対照

被検物質交付群と同一飼育条件のもとに被検物質を交付しない無処置対照群は平均体重 90.6 g のものが, 180 日後, 即ち実験終了日には 325.9 g の増加を示した. この間ラットは運動状態, 食欲, 毛並みの状態などに異常なく, 鼻出血, 眼変化も見られず, 極めて良好であつた.

この無処置の体重曲線とし, また一般状態を基準とし, 試料交付の各群を比較検索した (第3表, 第1及び8図).

(2) 第2群：1% C.P. 含有飼料交付の場合

ラットの体重は試料交付開始時平均体重 92.1 g のものが, 180 日後 338.9 g に増加した. その間体重曲線は逐日的に増加し, 順調な発育を示した. 体重曲線中 103 日目に全例一過性の体重減少を来たしているが, これは給水洩れによるもので, それ以後の経過に異常なく, 一般状態においても無処置対照群と比較して特に指摘すべき異常は認められず, むしろ 90 日以後はその平均値は無処置対照群より良好であつた (第4表, 第2及び8図).

(3) 第3群：3% C.P. 含有飼料交付の場合

ラットの体重は試料交付開始後逐日的に増加し, 10 匹の平均体重は試料交付開始時の 93.0 g より 180 日後 342.2 g に増加した. この間全く異常は認められず, 2例のみ 80 日から 118 日にかけて著しい体重の増加を現わした. 平均体重曲線は無処置対照より良好で, 且つ, 本群は C.P. 交付群中最もよい成長率を示した (第5表, 第3及び8図).

(4) 第4群：6% C.P. 含有飼料交付の場合

ラットの体重は試料交付開始後も逐日的に増加し, 良好な発育を示した. 10 匹の平均体重は試料交付開始後の 92.2 g より 180 日後 337.8 g に増加した. この間病的症状の発現は認められず, 微かに 50 日を経過してから個々の体重に増加の著しいものと普通の発育を示したものとの間に差が生じ, 曲線は上弦と下弦の間にやや巾が認められるようになつた. 併し乍ら平均体重は無処置対照群より良好で, 1% C.P. よりもむしろ上回る発育を示した (第6表, 第4及び8図).

(5) 第5群：1% S.P. 含有飼料交付の場合

ラットの体重は試料交付開始も逐日的増加を示し, 10 匹の平均体重は試料交付開始時の 89.7 g より 180 日後 348.5 g に増加した. この間病的乃至異常所見は認められず, 無処置対照群と比較してそれより一層発育よく, ただ1例のみ 28 日頃からやや増加の低い傾向を示した. 60 日頃には恢復し, その後は順調であつた (第7表, 第5及び8図).

(6) 第6群：3% C.P. 含有飼料交付の場合

ラットの体重は試料交付開始後も順調に増加し, 10 匹の平均体重は試分交付開始時の 92.9 g より 180 日後 359.1 g に増加した. この間何らの異常も認められず, この平均曲線は無処理対照群と比較して最もよく, 特に 87 日頃からの上昇が著しかつた (第8表, 第6及び8図).

(7) 第7群：6% S.P. 含有飼料交付の場合

ラットの体重は試料交付開始後も順調に逐日的増加を示し, 10 匹の平均体重は試料交付開始時の 93.4 g より 180日後 328.9 g に増加した. この間病的症状の発現なく, 一般状態も無処置対照群と比較して相異を示さなかつた. 1% 及び 3% S.P. 交付と比較した場合, その平均曲線はやや劣るが, 一般状態は全く変らなかつた (第9表, 第7及び8図).

2) C.P. 及び S.P. 交付ラットの血液一般に就いての検索

第181日に C.P. 及び S.P. の各試料交付群並びに無処置対照群全ラットの血液一般の検索を行つた. その結果は第10表の如くである (第10表).

試料交付の場合と対照との間には殆んど有無の差異は認められず, 特に赤血球及び白血球像に於いて変形乃至異常所見なく, 且つその数値も生理的動揺の範囲にとどまり, その他, 色素量並びに白血球百分率にも異常乃至病的所見は認められなかつた (第10表).

3) C.P. 及び S.P. 交付ラットの臓器重量及び容積に就いての検索

180 日間試料交付を行なつたラットを実験終了後第181 日に全例失血致死せしめて, 脳, 甲状腺, 副腎, その他内臓諸臓器の重量及び容積を測定した結果は第11表に示す如くである (第11表).

試料交付の場合と対照との間には有意の差異は認められなかつた.

4) C.P. 及び S.P. 交付ラットの病理組織学的検索

是等試料交付ラットの諸臓器について, 更に病理組織学的検索を行い, 各臓器に炎症, 変性或は腫瘍の発生等の病的異常所見の有無を厳格に検査した. 第9図は試料交付ラット臓器の組織像の一部である (第9図).

即ち, 被験全ラットは血液検査, 臓器重量及び容積の測定後, 肉眼的に各臓器の病的所見の有無を検索し,

Table 11

第11表 Calcium Propionate 及び Sodium Propionate を連続180日間交付せる場合の各群ラットの第181日の内臓諸臓器の重量（g）容積（ml）

ラット群別	番号	性別	体重(g)	大脳 重量(g)	大脳 容積(ml)	小脳 重量(g)	小脳 容積(ml)	甲状腺 重量(g)	甲状腺 容積(ml)	胃 重量(g)	胃 容積(ml)	心臓 重量(g)	心臓 容積(ml)	肝臓 重量(g)	肝臓 容積(ml)	脾臓 重量(g)	脾臓 容積(ml)	腎臓 右 重量(g)	腎臓 右 容積(ml)	腎臓 左 重量(g)	腎臓 左 容積(ml)	副腎 右 重量(g)	副腎 右 容積(ml)	副腎 左 重量(g)	副腎 左 容積(ml)	睾丸 右 重量(g)	睾丸 右 容積(ml)	睾丸 左 重量(g)	睾丸 左 容積(ml)	肺 重量(g)
I 無処置	No. 1	♂	304	1.27	1.23	0.54	0.53	0.04	0.02	1.54	1.48	1.15	1.09	10.3	9.5	1.34	1.20	1.00	0.92	1.00	0.93	0.02	0.01	0.03	0.02	1.27	1.22	1.34	1.29	1.75
	No. 2	♀	378	1.34	1.27	0.50	0.45	0.04	0.02	1.91	1.84	1.60	1.54	13.6	12.7	0.96	0.91	1.47	1.41	1.45	1.40	0.02	0.01	0.02	0.01	1.36	1.32	1.18	1.14	2.62
	No. 3	♀	332	1.08	1.05	0.51	0.49	0.04	0.02	1.83	1.79	1.25	1.20	11.5	10.7	0.86	0.83	1.09	1.02	1.10	1.06	0.02	0.01	0.02	0.01	1.31	1.27	1.33	1.30	1.89
	No. 4	♀	286	1.12	1.08	0.46	0.42	0.04	0.02	1.60	1.52	1.29	1.24	11.0	10.3	0.80	0.76	1.06	1.02	1.03	1.00	0.03	0.02	0.02	0.01	1.42	1.38	1.51	1.47	1.72
	No. 5	♀	300	1.10	1.08	0.50	0.49	0.04	0.02	1.60	1.54	1.24	1.22	10.6	10.0	1.10	1.04	1.09	1.04	1.14	1.10	0.02	0.01	0.02	0.01	1.26	1.23	1.28	1.26	1.84
	No. 6	♀	324	1.32	1.30	0.41	0.40	0.04	0.02	2.00	1.93	1.22	1.20	10.0	9.3	0.65	0.63	1.24	1.18	1.18	1.14	0.02	0.01	0.02	0.01	1.24	1.23	1.30	1.28	1.92
	No. 7	♀	389	0.91	0.89	0.46	0.44	0.04	0.02	1.84	1.80	1.40	1.36	11.0	10.2	0.87	0.83	1.15	1.11	1.06	1.02	0.02	0.01	0.02	0.01	1.12	1.00	1.18	1.10	1.88
	No. 8	♀	312	1.22	1.19	0.44	0.42	0.04	0.02	1.60	1.58	1.12	1.05	10.0	9.0	0.69	0.67	1.07	1.01	0.98	0.92	0.02	0.01	0.02	0.01	1.44	1.40	1.44	1.37	1.77
	No. 9	♀	339	1.28	1.22	0.55	0.53	0.04	0.02	2.20	2.07	1.20	1.13	11.2	10.5	1.05	0.99	1.24	1.20	1.24	1.20	0.02	0.01	0.02	0.01	1.39	1.32	1.40	1.34	1.80
	No. 10	♀	295	1.24	1.19	0.20	0.19	0.04	0.02	1.55	1.54	1.13	1.08	8.7	8.1	0.97	0.93	1.00	0.96	0.97	0.93	0.02	0.01	0.02	0.01	1.30	1.25	1.31	1.28	1.91
	平均		325.9	1.19	1.15	0.46	0.44	0.04	0.02	1.78	1.71	1.26	1.21	10.8	10.1	0.93	0.88	1.14	1.09	1.12	1.09	0.02	0.01	0.02	0.01	1.30	1.25	1.31	1.28	2.20
II C.P. 1%	No. 1	♂	315	1.31	1.25	0.35	0.33	0.04	0.02	2.00	1.97	1.24	1.21	10.0	9.3	1.05	1.02	1.24	1.18	1.17	1.13	0.02	0.01	0.02	0.01	1.13	1.09	1.11	1.07	2.20
	No. 2	♀	340	1.15	1.11	0.40	0.38	0.04	0.02	1.91	1.87	1.61	1.55	9.0	8.4	0.91	0.87	1.11	1.09	1.08	1.04	0.02	0.01	0.02	0.01	1.19	1.15	1.16	1.12	2.10
	No. 3	♀	311	1.20	1.17	0.54	0.52	0.04	0.02	1.75	1.73	1.35	1.28	11.3	10.5	0.82	8.77	1.09	1.03	1.14	1.08	0.02	0.01	0.02	0.01	1.40	1.35	1.42	1.37	1.60
	No. 4	♀	336	1.08	1.04	0.40	0.38	0.04	0.02	2.30	2.21	1.35	1.27	10.0	9.4	1.00	0.95	1.10	1.03	1.15	1.08	0.04	0.03	0.02	0.01	1.15	1.08	1.10	1.09	2.80
	No. 5	♀	341	1.24	1.20	0.52	0.50	0.04	0.02	2.20	1.98	1.50	1.46	11.2	10.4	0.94	0.87	1.35	1.29	1.34	1.27	0.02	0.01	0.03	0.02	1.31	1.25	1.35	1.25	3.20
	No. 6	♀	328	1.26	1.21	0.48	0.46	0.04	0.02	2.10	2.02	1.28	1.21	10.5	9.8	0.32	0.27	1.07	1.01	1.08	1.01	0.03	0.02	0.02	0.01	1.22	1.19	1.26	1.22	2.00
	No. 7	♀	364	1.20	1.14	0.43	0.41	0.04	0.02	1.55	1.52	1.36	1.30	10.4	9.8	2.30	2.22	1.10	1.04	1.15	1.09	0.02	0.01	0.02	0.01	1.30	1.26	1.29	1.24	2.20
	No. 8	♀	353	1.14	1.10	0.40	0.36	0.04	0.02	1.82	1.74	1.34	1.29	10.9	10.1	0.93	0.89	1.28	1.22	1.27	1.20	0.02	0.01	0.02	0.01	1.46	1.42	1.52	1.48	1.45
	No. 9	♀	371	1.27	1.22	0.55	0.53	0.04	0.02	1.98	1.94	1.60	1.52	11.2	10.5	0.93	0.89	1.28	1.22	1.27	1.20	0.02	0.01	0.02	0.01	1.58	1.54	1.60	1.56	2.50
	No. 10	♀	329	1.34	1.30	0.54	0.52	0.04	0.02	1.76	1.71	1.39	1.34	10.2	9.5	1.01	0.96	1.16	1.11	1.14	1.11	0.02	0.01	0.02	0.01	1.18	1.15	1.22	1.19	2.00
	平均		338.8	1.22	1.17	0.46	0.44	0.04	0.02	1.94	1.87	1.40	1.34	10.5	9.8	1.02	0.98	1.15	1.10	1.17	1.11	0.02	0.01	0.02	0.01	1.30	1.25	1.30	1.26	2.21
III C.P. 3%	No. 1	♂	323	1.22	1.16	0.44	0.42	0.04	0.02	1.62	1.56	1.22	1.17	11.0	10.1	0.84	0.80	1.12	1.05	1.04	0.98	0.02	0.01	0.02	0.01	1.38	1.34	1.60	1.55	1.90
	No. 2	♀	358	1.20	1.16	0.53	0.51	0.04	0.02	1.88	1.84	1.30	1.27	11.5	10.7	0.80	0.76	1.22	1.16	1.23	1.18	0.02	0.01	0.02	0.01	1.30	1.26	1.31	1.27	1.88
	No. 3	♀	353	1.30	1.28	0.55	0.53	0.04	0.02	1.78	1.54	1.25	1.23	9.3	8.6	0.76	0.74	1.00	0.95	1.00	0.95	0.02	0.01	0.02	0.01	1.56	1.51	1.54	1.49	4.20
	No. 4	♀	379	1.32	1.27	0.46	0.44	0.04	0.02	2.20	2.14	1.38	1.32	13.2	12.4	0.98	0.93	1.56	1.49	1.56	1.49	0.02	0.01	0.02	0.01	1.42	1.39	1.46	1.42	2.40
	No. 5	♀	320	1.23	1.18	0.54	0.52	0.04	0.02	1.40	1.35	1.22	1.17	8.8	8.2	0.80	0.78	1.06	1.02	1.12	1.07	0.02	0.01	0.02	0.01	1.46	1.42	1.45	1.40	2.40
	No. 6	♀	376	1.20	1.14	0.43	0.42	0.04	0.02	2.00	1.96	1.28	1.19	15.0	14.0	1.07	1.02	1.37	1.32	1.29	1.24	0.03	0.02	0.02	0.01	1.20	1.18	1.23	1.20	2.60
	No. 7	♀	338	1.02	1.00	0.40	0.39	0.04	0.02	1.58	1.56	1.15	1.12	10.5	9.8	0.90	0.86	1.10	1.08	1.15	1.12	0.02	0.01	0.02	0.01	1.46	1.42	1.45	1.40	2.40
	No. 8	♀	332	1.30	1.26	0.24	0.23	0.04	0.02	1.42	1.40	1.10	1.08	11.2	10.5	0.80	0.78	0.97	0.93	0.98	0.94	0.02	0.01	0.02	0.01	1.36	1.32	1.34	1.30	2.50
	No. 9	♀	337	1.14	1.09	0.42	0.40	0.04	0.02	1.48	1.42	1.24	1.20	10.9	10.1	1.28	1.23	1.13	1.08	1.18	1.13	0.02	0.01	0.02	0.01	1.13	1.09	1.11	1.07	2.50
	No. 10	♀	306	1.14	1.12	0.45	0.43	0.04	0.02	1.50	1.46	1.15	1.11	10.5	9.9	1.20	1.15	1.06	1.02	1.13	1.09	0.03	0.02	0.02	0.01	1.24	1.20	1.25	1.21	2.80
	平均		342.2	1.21	1.17	0.45	0.43	0.04	0.02	1.67	1.62	1.33	1.19	11.2	10.3	0.94	0.91	1.16	1.11	1.17	1.12	0.02	0.01	0.02	0.01	1.34	1.30	1.37	1.32	2.51

（第21巻第3・4合併号）

（1963年7月）

ラット群別	番号	性別	体重(g)	大脳 重量(g)	大脳 容積(ml)	小脳 重量(g)	小脳 容積(ml)	甲状腺 重量(g)	甲状腺 容積(ml)	胃 重量(g)	胃 容積(ml)	心臓 重量(g)	心臓 容積(ml)	肝臓 重量(g)	肝臓 容積(ml)	脾臓 重量(g)	脾臓 容積(ml)	腎臓 右 重量(g)	腎臓 右 容積(ml)	腎臓 左 重量(g)	腎臓 左 容積(ml)	副腎 右 重量(g)	副腎 右 容積(ml)	副腎 左 重量(g)	副腎 左 容積(ml)	睾丸 右 重量(g)	睾丸 右 容積(ml)	睾丸 左 重量(g)	睾丸 左 容積(ml)	肺 重量(g)
IV C.P. 6%	No. 1	♂	365	1.24	1.18	0.54	0.52	0.04	0.02	1.74	1.71	1.52	1.46	13.4	12.5	1.00	0.97	1.26	1.22	1.34	1.30	0.02	0.01	0.02	0.01	1.28	1.24	1.28	1.24	3.20
	No. 2	♀	354	1.32	1.28	0.46	0.44	0.04	0.02	1.61	1.57	1.30	1.28	13.7	12.8	0.91	0.89	1.18	1.14	1.10	1.04	0.02	0.01	0.02	0.01	1.10	1.06	1.10	1.06	2.50
	No. 3	♀	336	1.24	1.19	0.48	0.46	0.04	0.02	1.65	1.61	1.29	1.25	12.2	11.3	1.09	1.03	1.20	1.16	1.19	1.15	0.02	0.01	0.02	0.01	1.31	1.27	1.40	1.36	2.80
	No. 4	♀	302	1.16	1.12	0.51	0.49	0.04	0.02	1.54	1.50	1.56	1.49	10.5	9.3	1.01	0.97	1.25	1.19	1.32	1.26	0.02	0.01	0.02	0.01	1.38	1.33	1.43	1.38	3.00
	No. 5	♀	334	1.15	1.13	0.54	0.53	0.04	0.02	1.78	1.76	1.32	1.29	12.0	11.2	0.78	0.75	1.24	1.21	1.20	1.15	0.02	0.01	0.02	0.01	1.54	1.51	1.50	1.47	3.80
	No. 6	♀	332	1.12	1.07	0.50	0.49	0.04	0.02	1.55	1.50	1.28	1.22	11.3	10.5	0.79	0.76	1.03	1.00	1.06	1.03	0.02	0.01	0.02	0.01	1.18	1.14	1.26	1.22	3.20
	No. 7	♀	371	1.31	1.27	0.46	0.44	0.04	0.02	2.20	2.15	1.40	1.35	12.9	12.1	1.15	1.10	1.35	1.29	1.30	1.24	0.02	0.01	0.02	0.01	1.66	1.61	1.60	1.55	3.20
	No. 8	♀	354	1.28	1.24	0.34	0.32	0.04	0.02	1.80	1.75	1.60	1.58	13.2	12.4	1.24	1.16	1.35	1.31	1.38	1.34	0.02	0.01	0.02	0.01	1.54	1.50	1.56	1.52	2.90
	No. 9	♀	339	1.15	1.12	0.38	0.36	0.04	0.02	1.76	1.73	1.33	1.29	11.9	11.0	1.04	1.02	1.24	1.19	1.16	1.11	0.02	0.01	0.02	0.01	1.26	1.22	1.29	1.25	2.40
	No. 10	♀	299	1.24	1.19	0.50	0.48	0.04	0.02	1.52	1.49	1.24	1.18	10.2	9.5	0.70	0.68	1.06	1.01	1.12	1.07	0.03	0.02	0.02	0.01	1.14	1.09	1.15	1.11	2.40
	平均		337.8	1.22	1.18	0.47	0.45	0.04	0.02	1.72	1.68	1.39	1.34	12.2	12.3	0.97	0.93	1.22	1.17	1.22	1.17	0.02	0.01	0.02	0.01	1.34	1.30	1.36	1.32	2.94
V S.P. 1%	No. 1	♂	358	1.10	1.07	0.52	0.50	0.04	0.02	2.00	1.60	1.50	1.45	12.4	11.5	1.16	1.10	1.40	1.34	1.48	1.42	0.02	0.01	0.02	0.01	1.28	1.26	1.32	1.30	2.50
	No. 2	♀	317	1.40	1.34	0.55	0.53	0.04	0.02	0.87	0.82	1.25	1.21	10.2	9.3	1.08	1.04	1.25	1.18	1.20	1.15	0.03	0.02	0.03	0.02	1.52	1.49	1.55	1.51	2.00
	No. 3	♀	336	1.30	1.27	0.25	0.23	0.04	0.02	1.65	1.61	1.05	0.99	10.4	9.7	0.48	0.42	0.98	0.92	1.15	0.99	0.02	0.01	0.02	0.01	1.05	1.02	0.94	0.91	1.40
	No. 4	♀	366	1.30	1.23	0.25	0.22	0.04	0.02	2.00	1.93	1.40	1.32	12.8	11.9	1.08	1.05	1.25	1.19	1.25	1.19	0.02	0.01	0.02	0.01	1.18	1.13	1.20	1.15	2.50
	No. 5	♀	312	1.17	1.11	0.75	0.71	0.04	0.02	1.97	1.89	1.42	1.38	11.5	10.8	0.68	0.62	1.30	1.24	1.32	1.26	0.03	0.01	0.03	0.02	1.47	1.44	1.24	1.20	2.00
	No. 6	♀	365	1.34	1.30	0.52	0.50	0.04	0.02	1.70	1.68	1.27	1.23	10.8	10.0	0.65	0.63	1.18	1.13	1.35	1.30	0.02	0.02	0.02	0.01	1.22	1.18	1.22	1.18	2.00
	No. 7	♀	369	1.25	1.21	0.55	0.53	0.04	0.02	1.82	1.78	1.70	1.62	12.8	11.9	1.05	1.03	1.26	1.22	1.20	1.16	0.02	0.01	0.02	0.01	1.22	1.19	1.25	1.22	3.20
	No. 8	♀	376	1.30	1.27	0.50	0.48	0.04	0.02	1.76	1.73	1.30	1.25	11.5	10.7	0.74	0.72	1.18	1.16	1.25	1.23	0.03	0.02	0.02	0.01	1.35	1.32	1.44	1.41	2.30
	No. 9	♀	305	1.18	1.15	0.50	0.48	0.04	0.02	1.92	1.88	1.28	1.24	10.2	9.6	1.00	0.99	1.32	1.30	1.20	1.18	0.02	0.01	0.02	0.01	1.18	1.16	1.18	1.16	2.20
	No. 10	♀	356	1.25	1.21	0.32	0.31	0.04	0.02	1.56	1.52	1.52	1.46	10.8	10.0	1.34	1.28	1.32	1.26	1.38	1.32	0.02	0.01	0.02	0.01	1.46	1.41	1.50	1.45	2.10
	平均		348.5	1.26	1.22	0.47	0.45	0.04	0.02	1.73	1.64	1.37	1.32	11.3	10.5	0.93	0.89	1.24	1.19	1.28	1.22	0.02	0.01	0.02	0.01	1.15	1.26	1.28	1.25	2.17
VI S.P. 3%	No. 1	♂	326	1.10	1.06	0.45	0.44	0.04	0.02	1.44	1.38	1.44	1.37	9.5	8.9	0.90	0.86	1.15	1.10	1.15	1.10	0.02	0.01	0.02	0.01	1.46	1.42	1.45	1.41	6.00
	No. 2	♀	348	1.27	1.22	0.55	0.53	0.04	0.02	1.76	1.70	1.40	1.36	12.8	11.9	0.84	0.81	1.35	1.30	1.34	1.29	0.02	0.01	0.02	0.01	1.42	1.38	1.45	1.37	2.00
	No. 3	♀	378	1.14	1.10	0.30	0.29	0.04	0.02	1.80	1.76	1.40	1.33	11.4	10.6	0.96	0.93	1.20	1.16	1.13	1.08	0.02	0.01	0.03	0.02	1.15	1.09	1.12	1.06	2.60
	No. 4	♀	370	1.10	1.05	0.38	0.37	0.04	0.02	1.52	1.45	1.29	1.22	11.1	10.9	0.50	0.46	1.22	1.17	1.18	1.13	0.02	0.01	0.02	0.01	1.25	1.20	1.20	1.15	1.50
	No. 5	♀	343	1.22	1.17	0.42	0.40	0.04	0.02	1.80	1.78	1.30	1.28	12.0	11.3	0.98	0.93	1.15	1.10	1.20	1.16	0.03	0.02	0.03	0.02	1.35	1.31	1.34	1.30	2.00
	No. 6	♀	400	1.17	1.16	0.42	0.40	0.04	0.02	1.86	1.79	1.20	1.12	13.4	12.6	1.16	1.13	1.10	1.04	1.20	1.14	0.03	0.02	0.03	0.02	1.40	1.36	1.36	1.32	2.00
	No. 7	♀	367	1.26	1.21	0.45	0.43	0.04	0.02	1.92	1.89	1.35	1.28	14.6	13.6	0.75	0.71	1.35	1.30	1.32	1.27	0.02	0.01	0.02	0.01	0.75	0.70	0.74	0.69	3.90
	No. 8	♀	403	1.28	1.24	0.51	0.49	0.04	0.02	2.00	1.94	1.48	1.41	14.4	13.6	0.89	0.87	1.36	1.30	1.42	1.35	0.03	0.02	0.02	0.01	1.34	1.30	1.38	1.34	2.50
	No. 9	♀	337	1.22	1.19	0.43	0.42	0.04	0.02	1.58	1.56	1.18	1.13	10.8	10.1	1.20	1.14	1.18	1.12	1.10	1.04	0.03	0.02	0.02	0.01	1.35	1.29	1.32	1.26	2.20
	No. 10	♀	359	1.30	1.26	0.40	0.39	0.04	0.02	2.40	2.48	1.30	1.26	13.4	13.0	0.96	0.94	1.32	1.27	1.34	1.28	0.03	0.02	0.02	0.01	1.18	1.14	1.20	1.14	2.30
	平均		359.1	1.21	1.17	0.43	0.42	0.04	0.02	1.81	1.77	1.33	1.28	12.3	11.6	0.91	0.88	1.24	1.19	1.24	1.18	0.03	0.02	0.02	0.01	1.27	1.22	1.26	1.20	2.70
VII S.P. 6%	No. 1	♂	320	1.16	1.12	0.42	0.40	0.04	0.02	1.64	1.57	1.25	1.19	14.2	13.2	1.06	1.00	1.32	1.25	1.34	1.31	0.03	0.02	0.02	0.01	1.32	1.27	1.28	1.23	1.60
	No. 2	♀	337	1.14	1.10	0.48	0.47	0.04	0.02	1.84	1.79	1.28	1.22	12.0	11.2	0.65	0.61	1.16	1.12	1.16	1.12	0.02	0.01	0.02	0.01	1.15	1.10	1.19	1.14	2.00
	No. 3	♀	329	1.15	1.09	0.50	0.48	0.04	0.02	1.64	1.60	1.13	1.08	12.0	11.1	0.60	0.57	1.28	1.23	1.16	1.10	0.02	0.01	0.03	0.02	1.14	1.10	1.10	1.06	2.00
	No. 4	♀	322	1.24	1.18	0.40	0.38	0.04	0.02	1.74	1.72	1.28	1.22	12.2	11.3	0.74	0.68	1.36	1.29	1.37	1.30	0.02	0.01	0.02	0.01	1.72	1.66	1.72	1.66	2.40
	No. 5	♀	330	1.18	1.12	0.46	0.45	0.04	0.02	1.75	1.70	1.28	1.22	11.5	10.7	0.84	0.80	1.15	1.09	1.10	1.04	0.02	0.01	0.02	0.01	1.30	1.25	1.35	1.30	2.00
	No. 6	♀	340	1.26	1.21	0.51	0.49	0.04	0.02	1.70	1.67	1.36	1.29	11.0	10.2	0.95	0.88	1.23	1.17	1.35	1.29	0.02	0.01	0.02	0.01	1.29	1.24	1.30	1.26	2.50
	No. 7	♀	328	1.16	1.10	0.46	0.44	0.04	0.02	1.62	1.55	2.30	2.21	10.4	9.7	0.80	0.76	1.06	1.02	1.05	1.00	0.03	0.02	0.02	0.01	1.23	1.19	1.20	1.16	2.20
	No. 8	♀	337	1.20	1.16	0.47	0.45	0.04	0.02	2.20	2.14	1.58	1.52	11.5	10.6	0.82	0.77	1.52	1.45	1.46	1.39	0.02	0.01	0.02	0.01	1.25	1.21	1.27	1.23	2.00
	No. 9	♀	346	1.12	1.08	0.44	0.42	0.04	0.02	1.88	1.87	1.33	1.26	12.0	11.1	1.00	0.95	1.24	1.19	1.26	1.21	0.02	0.01	0.02	0.01	1.20	1.16	1.25	1.21	1.80
	No. 10	♀	300	1.18	1.13	0.42	0.39	0.04	0.02	1.64	1.57	1.12	1.07	8.6	8.5	0.84	0.79	1.02	0.97	0.95	0.88	0.02	0.01	0.02	0.01	1.14	1.10	1.08	1.01	2.20
	平均		328.9	1.18	1.13	0.46	0.44	0.04	0.02	1.77	1.72	1.40	1.33	11.5	10.8	0.83	0.73	1.23	1.18	1.22	1.16	0.02	0.01	0.02	0.01	1.27	1.23	1.27	1.23	2.07

Fig 1 第1図　無処置対照ラットの体重曲線

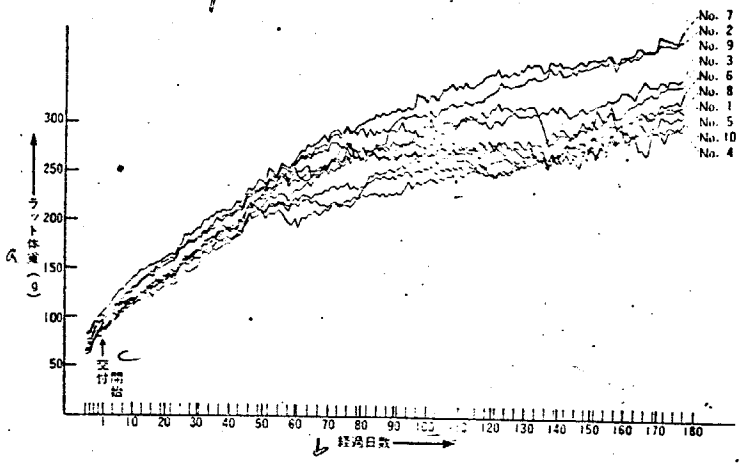
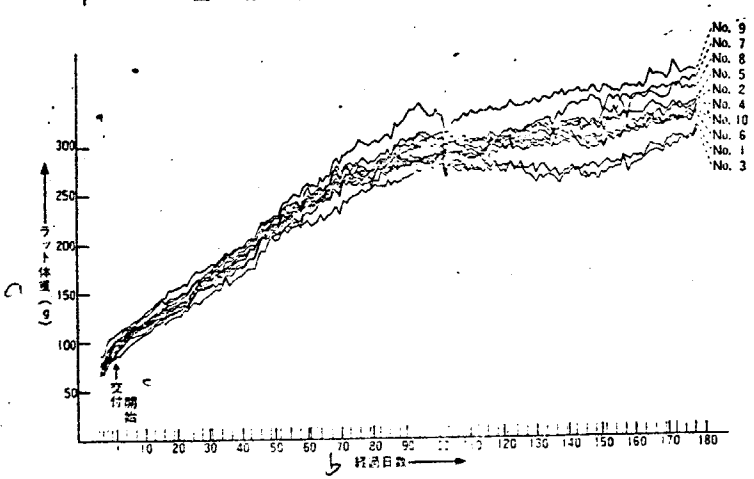


Fig 2 第2図　1% Calcium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重曲線



然る後各臓器をホルマリンで固定し，Hematoxylin-Eosin 染色を行つて病理組織学的検索を行つた．

個々の臓器組織所見に就いては，食道に於いては上皮細胞及び粘膜下組織に異常なく，正常像を示した．胃に於いては腺構造整然として消耗症状認められず，脂肪変性，石灰沈着なく，上皮細胞や皮下組織に異常所見はみられず，萎縮性或は肥厚性の炎症もみられなかつた．小腸及び大腸に於いては上皮細胞並びに腺細胞に腫大，萎縮或は壊死等の退行性変化なく，粘膜固有層の自由細胞の増加も認められず，粘膜下組織にも充血，血栓，出血或は水腫等の循環障碍を思わせる病的異常所見は認められなかつた．肝臓に於いては肝細胞に脂肪変性及び壊死等の退行性変化なく，且つ炎症像を欠き，肝組織の正常構造は完全に保有されていた．腎臓に於いては腎組織に萎縮，溷濁，腫脹乃至脂肪沈着なく，壊死性の変化も認められなかつた．特に糸球体

Fig 3 第3図　3% Calcium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重曲線

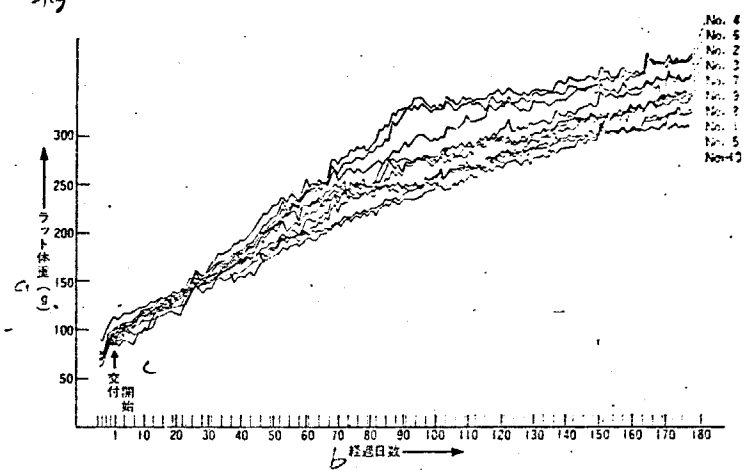
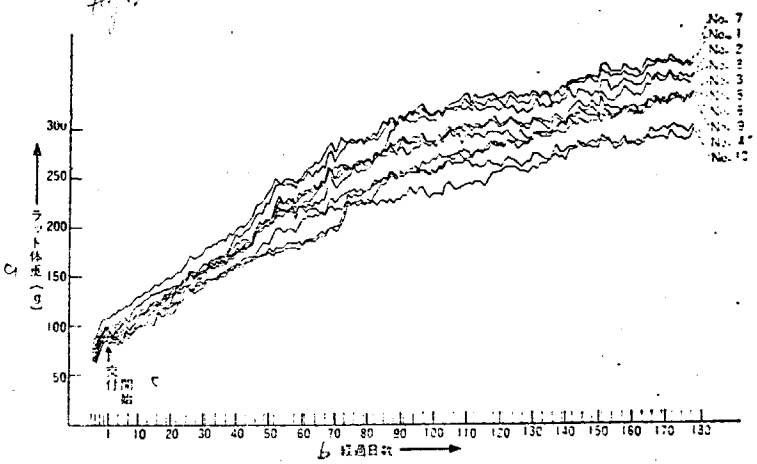
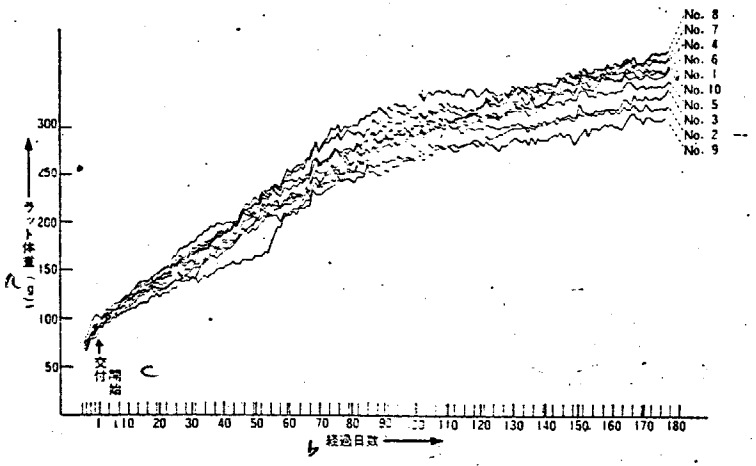


Fig 4 第4図　6% Calcium Propionate 含有飼料をラットに連続180日間経口交付せる場合の体重曲線

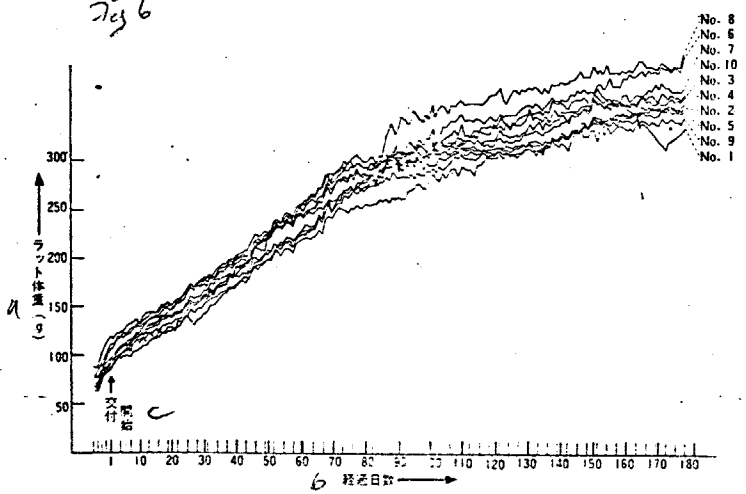


には充血，腫脹或は退行性病変等の病的所見なく，又，細尿管上皮にも変性は見られず，円柱形成の異常も認められなかつた．脾臓に於いては微かに hemosiderin 沈着を認めた例もあるが，これは健常動物にも時にみられるもので生理的と考えてよく，むしろ問題視すべき組胞増加，充血等の異常，沪胞腫大等の病的像は観察されなかつた．心臓に於いては脂肪変性，石灰沈着或は実質性の溷濁等の退行性病変は認められず，うつ血，水腫乃至出血はなく，血栓形成による循環障碍もみられなかつた．尚心内膜の炎症性変化，心筋の炎症等の炎症性病的変化も認められなかつた．肺臓に於いては程度の気管支炎の症状を認めた少数例はあるが，この症状は対照群中にも認められるところから被検物質による病的異常とは考えられない．その他大多

第5図　1% Sodium Propionate 含有飼料をラットに連続 180 日間経口交付せる場合の体重曲線



第6図　3% Sodium Propionate 含有飼料をラットに連続 180 日間経口交付せる場合の体重曲線



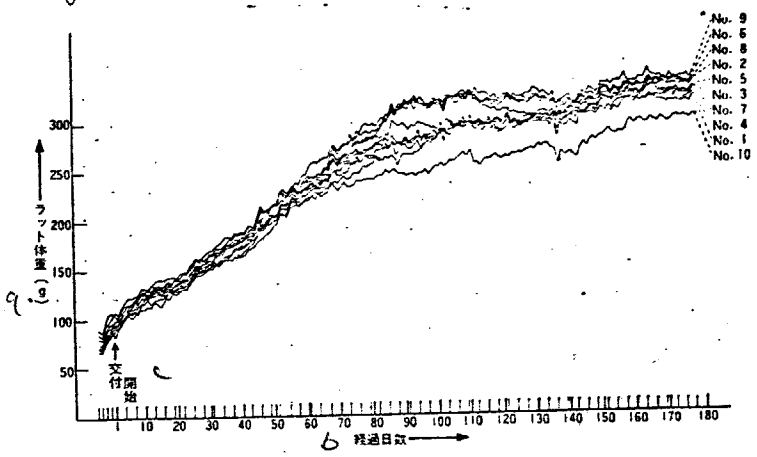
数例は肺組織中に腫瘍形成乃至壊疽性変化なく，動脈性充血，うっ血又は出血等の異常所見認められず，且つ循環障碍も認められなかつた．その他臓に於いては発育障碍なく，炎症，変性乃至腫瘍の発現等全く認められず，腎臓に於いても炎症性変乃至循環障碍等認められなかつた．甲状腺に於いては濾胞肥大のみられたものもあり，とくに鉄染色を施行したが，これは対照群中にもみられたところから，被検物質交付による病的異常とは考えらない．副腎，睾丸に於いては何等の病的変化は認められなかつた．

(B)　一般薬理作用に関する検索

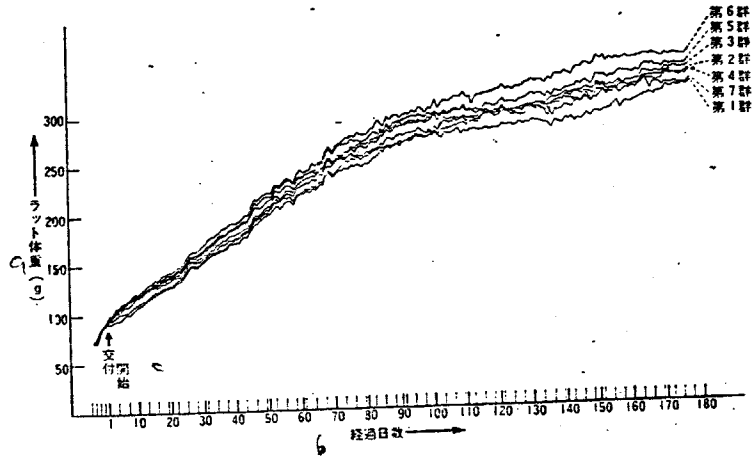
1.　C.P. 及び S.P. のウサギ呼吸及び血圧に対する作用

Urethane に依り麻酔したウサギの呼吸及び頸動脈

第7図　6% Sodium Propionate 含有飼料をラットに連続 180 日間経口交付せる場合の体重曲線



第8図　Calcium Propionate 及び Sodium Propionate 各交付量に於けるラット体重曲線の比較（平均）



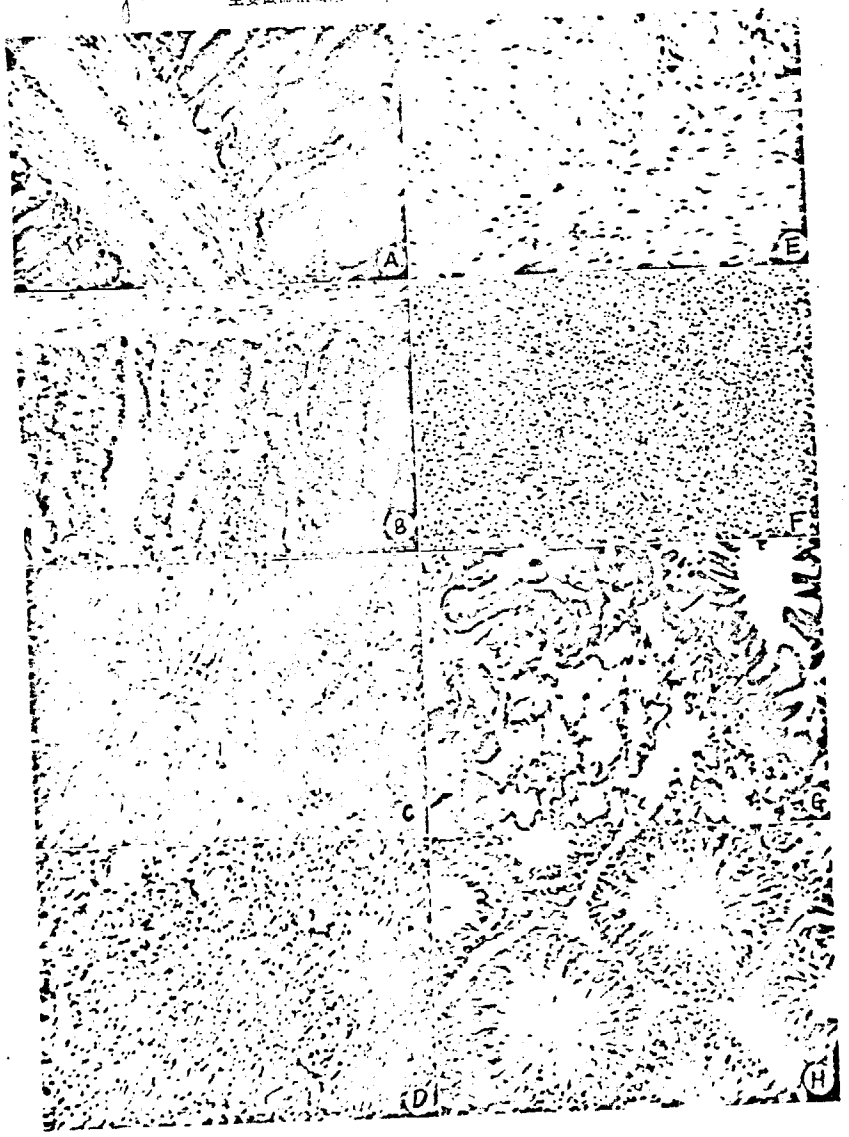
圧に対する作用を観察した．

C.P. 及び S.P. 共少量では呼吸及び血圧に殆んど変化なく，大量，即ち 500 mg/kg の静脈内交付で，C.P. は血圧が直ちに下降し，呼吸も一旦停止を来たし，やや快復するかにみえるが，数分後 Cheyne-Stokes 様呼吸を行い停止した．S.P. は血圧やや一過性に下降した．その際呼吸は一過性に興奮して振巾及び回数を増加した．尚，S.P. は C.P. の呼吸停止量である 500 mg/kg と同様量では呼吸停止を来たさなかつた．S.P. は 1,000 mg/kg で血圧降下し，呼吸停止を来たした（第 10,11 図）．

2.　C.P. 及び S.P. の刺出心臓及び心臓及び平滑筋に対する作用

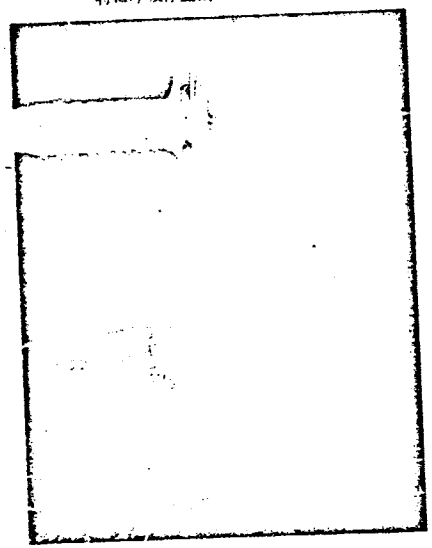
C.P. 及び S.P. の両者は刺出心臓及び平滑筋に対し特に著しい作用を現わさなかつた．強いて記せば両者の作用強度は C.P. の方が S.P. より強いように思

第9図　プロピオン酸塩（6% C.P.）を[illegible]第181日の主要臓器組織像

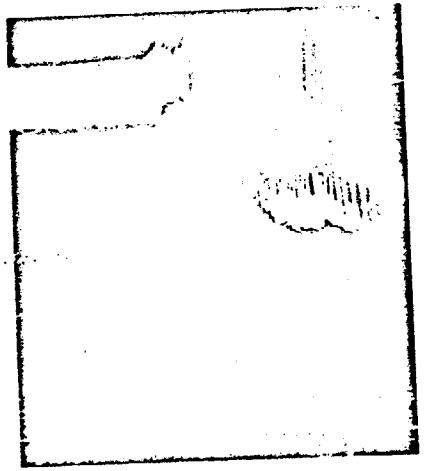


A：胃　B：小腸　C：肝臓　D：腎臓
E：心臓　F：脾臓　G：肺臓　H：睾丸

第10図　ウサギ呼吸血圧に対する S.P. の作用，特に呼吸停止系（S.P. 1,000 mg/kg i.v.）



第11図　ウサギ呼吸血圧に対する C.P. の作用，特に呼吸停止系（C.P. 500 mg/kg i.v.）



われる．

IV. 総括並びに考察

以上の実験結果を総括し，次の如く考察する．

1) マウスの急性実験に於いて，LD_{50} は，静脈内交付では C.P. は 580 mg/kg，S.P. は 1,380mg/kg であり，腹腔内交付では，C.P. は 720 mg/kg，S.P. は 2,250 mg/kg，であり，皮下交付では，C.P. は 1,020 mg/kg，S.P. は 3,200 mg/kg であり，経口交付では，C.P. は 3,340 mg/kg，S.P. は 5,100 mg/kg である．殊に経口交付では急性毒性の発現が C.P.，S.P. 共可成りの大量を要することから，S.P. 及び C.P. を含めたプロピオン酸塩の毒性は極めて低いか，皆無に近いものと推定される．

2) ラットに 180 日間試料を交付して，この間の7群のラット体重曲線を無処置飼育の対照群と比較観察すると，C.P. 及び S.P. の平均成長曲線と対照群の平均成長曲線との間には殆んど相違を認め難い．強いて記せば 180 日目の体重は 3% S.P. > 1% S.P. > 3% C.P. > 6% C.P. > 1% C.P. > 6% S.P. > Control（対照）の順位となり，何ずれの曲線も対照群の上位にある．又，C.P. と S.P. の相互関係については，C.P. は S.P. の間にあり，両者間の差異は認め難い．又，変異関係については有意の差異は現われていない．これら成長曲線と一般状態の観察から考察して，プロピオン酸塩の影響は認め難く，且つ毒性の発現症候は認められない．

3) 各諸量の C.P. 及び S.P. 交付ラットの血液所見は対照と比較して，赤血球数，白血球数，血色素量，色素指数に異常数値を示さず，白血球百分比も生理的動揺範囲内にあり，全く正常である．即ち，プロピオン酸塩は末梢血液に対し，何等の影響も及ぼさないものと思考される．

4) 各諸量の C.P. 及び S.P. 交付ラットの脳及び内臓諸臓器の重量及び容積を測定した結果は試料交付群と対照群との間に，概して著しい差異は認められず，これら臓器に対しプロピン酸塩は影響を及ぼさないものと思われる．

5) 上記のラット諸臓器は病理組織学的検索に於いて，正常の組織像を保有し，各臓器に炎症，変性，壊死及び腫瘍の発生等の病的異常所見は認められない．ごく一部の臓器組織に hemosiderin 沈着を認めたものもあり，甲状腺に濾胞拡大のあるものを認めたが，対照群中に同様症状のものがあることから，C.P. 乃至 S.P. の毒性によるものとは思考され難く，是等の病理組織学的検索に於いて，プロピオン酸塩の毒性は全く認められないものと思考される．

6) 一般薬理学的検索に於いて，プロピオン酸塩は特有の薬理作用を呈さず，呼吸及び血圧に対しても大量を要し，平滑筋にも殆んど作用を及ぼさない．即ち，C.P. 及び S.P. の一般作用は弱く，且つ毒性も

認め難い．

V. 本実験の結論並びに判定

プロピオン酸塩である Calcium Propionate 及び Sodium Propionate はマウスの急性毒性実験に於いて，経口交付では致死せしめるためには極めて大量を要する．即ち，毒性は極めて微少か，皆無に近いと認定される．

ラットの経口交付による慢性毒性実験に於いて，その体重曲線は Calcium Propionate 及び Sodium Propionate の両者共，対照群と差異がないか，むしろ優位の経過を示す．是等試料交付により，成長は阻止されず，順調な発育を送げ，全く毒性を現わさない．

更に血液所見，内臓諸臓器の重量及び容積も対照との間に有意の差異は認められない．又，是等ラットの諸臓器は病理組織学的検索に於いて，対照との間に何等の差異を認めない．

以上によりプロピオン酸塩，就中 Calcium Propionate 及び Sodium Propionate の毒性は皆無に等しいと判定する．

以上の実験結果を基礎とし，尚，信憑される文献等を併せ考察して，本物質は一定の食品に添加して使用し得るものと判定する．

尚，本実験中，病理組織学の検索は本学病理学主任大高裕一教授に依頼した．ここに感謝の意を表する．

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SOME ANTIBACTERIAL PROPERTIES OF SODIUM PROPIONATE

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Using the agar cup-plate technique, Keeney *et al.*[1] found that a propionate ointment compared very favourably with a 5 per cent. sulphathiazole ointment against *Staphylococcus aureus* and β-hæmolytic *Streptococcus*. More recently, Theodore[2,3] has listed groups of fungi and bacteria of which growth is inhibited by sodium propionate; these organisms include, Fungi: — *Trichophyton, Epidermophyton, Microsporum, Candida, Aspergillus, Pityrosp. ovale;* Bacteria:—*Staphylococcus aureus, Staphylococcus albus, Pneumococcus, Pseudomonas æruginosa, Escherichia coli, Streptococcus.*

Our investigations are concerned with a short-term assessment of the activity of sodium propionate against common pathogens, and with *in vitro* studies of the bacteriostatic efficiency of the compound, as compared with that of sulphacetamide sodium, employing pharmaceutical preparations in clinical use. This sulphonamide was selected since, like sodium propionate, it is widely used for topical application to infected areas.

General Micromology

Various micro-organisms were used in a short study of the activity of sodium propionate at different *p*H values, agar, broth and blood media being employed as applicable. A number of tests showed that the nature of the medium made little material difference to the results with any particular organism, and the presence of blood did not appear to affect the activity of sodium propionate, as may be seen from Table I.

TABLE I

Effect of Sodium Propionate on the Growth of *Pseudomonas æruginosa* and *Proteus vulgaris*

Growth after 48 hours

Organism	Medium	Sodium Proprionate per cent.			
		0	1	2·5	5
Pseudomonas æruginosa	Blood Agar	+ +	0	0	0
" "	Nutrient Broth	+ +	0	0	0
Proteus vulgaris	Nutrient Agar	+ +	+ +	+	0
"	Blood Agar	+ +	+ +	tr	0
"	Nutrient Broth	+ +	+ +	+	0

It was observed that the inhibition of fungi and bacteria increased as the *p*H passed from the alkaline to the acid side; this factor may be of

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considerable importance in clinical application owing to the reaction of the skin, nasal and vaginal mucosae and possibly other tissues. Examples of the effects of *p*H are provided in Table II.

TABLE II

EFFECT OF SODIUM PROPIONATE ON THE GROWTH OF *ASPERGILLUS NIGER*, *ASPERGILLUS FUMIGATUS*, AND *CANDIDA ALBICANS* MALT AGAR CULTURES AT *p*H 7, 6 AND 5; GROWTH AFTER 96 HOURS

	*p*H 7			*p*H 6			*p*H 5		
Sodium Propionate per cent.	0	0·5	1	0	0·5	1	0	0·5	1
Aspergillus niger	+ +	+	+	+ +	+	tr	+ +	0	0
Aspergillus fumigatus ...	+ +	+ +	+ +	+ +	+	+	+ +	+	tr
Candida albicans	+ +	+	tr	+	0	0	+	0	0

Staph. aureus, Ps. æruginosa, E. coli and *Proteus vulgaris* cultured on nutrient agar were similarly inhibited more readily by sodium propionate at *p*H 7 than at *p*H 8. The inhibitory concentrations for certain common bacteria after 48 hours at *p*H 7 may be summarised as follows:—

Organism	*Inhibitory Concentration of Sodium Propionate.* per cent.
Ps. æruginosa	approx. 1·0
S. typhosa	
Staph. aureus (Heatley)	2·5
Staph. aureus (Coagulase +ve)	> 2·5
Staph. aurant. (Coagulase −ve)	
Proteus vulgaris	
Dipl. pneumoniae	
E. coli	5·0
Strep. pyogenes	5·0

Marked inhibition was caused by 1 per cent. or less of sodium propionate in the case of neutral cultures of *S. typhosa, Ps. æruginosa* (complete suppression at about 1 per cent.), *Staph. aureus* and *Dipl. pneumoniæ*, but rather more than 1 per cent. was required to reduce appreciably the growth of *Proteus vulgaris, E. coli* and *Strep. pyrogenes*. In these general tests, we employed only a limited range of fungi, since the fungistatic and fungicidal properties of sodium propionate have been fully investigated by Theodore[3]; Peck and his associates[4,5,6,7], Keeney[8] and other workers.

SODIUM PROPIONATE COMPARED WITH SULPHACETAMIDE SODIUM

Although bacteriostatic activity *in vitro* may not parallel therapeutic efficiency, it was considered desirable to compare the effects of similar concentrations of sodium propionate and of sulphacetamide sodium against important pathogenic bacteria. For this series of tests, the following pharmaceutical preparations were used throughout:

(a) Sodium Propionate Solution 10 per cent.; *p*H approx. 7·2.
(b) Sulphacetamide Sodium Eye Drops N.F. 10 per cent.

Group 1. COMPARISON OF ACTIVITY

Organisms—Ps. aeruginosa, Staph. aureus (Heatley strain), *Staph. aureus* (Coagulase +ve), *Staph. aurantiacus* (Coagulase −ve).

Method—Quadruple strength nutrient broth was diluted with the test solutions, and sterile distilled water if necessary, to produce the desired concentrations of the drugs. The inoculant was added in the form of 1 drop of an 18-hour broth culture and the preparation was incubated at 37°C.

Results—Growth was copious in all control cultures. The inhibitory concentrations of the two agents may be generalised by means of a simple system of brackets at 0, 1, 2·5 and 5 per cent., as in Table III.

TABLE III

Organism	Complete Suppression after 72 Hours by	
	Sodium Propionate per cent.	Sulphacetamide Sodium per cent.
Ps. aeruginosa	< 1·0	< 1
Staph. aureus (Heatley)	< 2·5	> 1
Staph. aureus (Coagulase + ve)...	> 2·5	< 1
Staph. aurantiacus (Coagulase − ve)	> 2·5	> 1

Group 2. ACCLIMATISATION EXPERIMENTS

Organism—Staph. aureus (Heatley strain).

Method—The organism was grown at 37°C. for 4 days in nutrient broth containing 0·8 per cent. of sodium propionate or of sulphacetamide sodium. It was then subcultured for 4 days in broth with 1·25 per cent. of the separate drugs, and loopfuls were subsequently used to inoculate broth containing varying concentrations of the compounds. The resultant culture was incubated for a further period of 72 hours before estimating growth.

Results—Comparison with 8-day control cultures indicated that the organism did not become appreciably resistant to sodium propionate. There was, however, some evidence of acclimatisation to sulphacetamide sodium, since the treated culture exhibited multiplication in the presence of 3 per cent. of this drug.

Group 3. TEST FOR SYNERGISM OR ANTAGONISM

Organism—Staph. aureus (Heatley strain).

Method—The method adopted was similar to that employed in Group I, but in this test for synergism or antagonism the organism was cultured in broth containing various concentrations of a mixture of sodium propionate 50 per cent. and sulphacetamide sodium 50 per cent.

Results—Growth of the staphylococcus was evident after 24 hours in the presence of 4 per cent. of the mixture (i.e., 2 per cent. of sodium pro-

pionate + 2 per cent. of sulphacetamide sodium). Comparison with controls and reference to Table III thus indicate that some antagonism may exist between these drugs.

Interpretation of Results

Although the sulphonamides have some effect on certain strains of *Trichophyton, Aspergillus niger* and species of *Actinomyces*, their antifungal activity appears to be very limited, and many organisms, including common yeasts, are resistant to them. Solutions of sulphacetamide sodium, indeed, frequently form good growth media and the ready multiplication of moulds necessitates the use of preservatives, such as benzalkonium chloride U.S.P., which is probably superior to the esters of *p*-hydroxybenzoic acid for this purpose. Since it is apparent that sulphacetamide sodium has only a limited activity against a restricted range of fungi, we decided to omit the latter organisms from the comparative tests.

Sodium propionate becomes more active against bacteria and fungi as the media are made less alkaline or more acid, so indicating an enhanced efficiency at the *p*H of the vagina, nasal secretion and skin. Although it is generally believed that the activity of sulphonamide solutions depends upon the anion and that antibacterial efficiency may, within certain limits, increase in alkaline media, it would appear that the effects of *p*H vary with the drug and also with the organism under test. Solutions containing 10 per cent. of sulphacetamide sodium have *p*H about 9, and this value may be adjusted to approximately 7·4 with 0·1 per cent. of sulphacetamide, as described in the B.P.C., or to about 6·9 with boric acid, as in the case of the N.F. and B.P.C. eye drops. Further acidification, however, results in precipitation of sulphacetamide, and it was considered impracticable to compare, by our method, the activity of sulphacetamide sodium with that of sodium propionate at the *p*H of certain tissues.

Bigger[9] has shown that boric acid and sulphathiazole are mutually antagonistic, and has suggested that it may be inadvisable to use boric acid in conjunction with sulphonamides. We have not ascertained whether sulphacetamide sodium is similarly affected, but any disadvantage induced by antagonism is possibly offset by the use of certain media which may contain substances which potentiate sulphonamides. Winkler and Julius[10] stated that a sulphanilamide-activating principle is present in the red cells of horse blood, although not in the blood of man, sheep, rabbits or mice; on the other hand, Bigger and Ware[11] found that a sulphonamide-potentiating "L substance" is present in commercial meat essences and extracts, dehydrated beef and horseflesh, commercial yeast extracts, human serum and urine and hæmolysed human and horse red blood-cells, this "L substance" being different from the principle responsible for the Harper and Cawston effect.

Our tests are intended only to provide an indication of the respective clinical values of sodium propionate and sulphacetamide sodium pre-

parations in local infections, since sodium propionate is applied topically to the eyes, ears, nose, vagina, skin and other tissues, but it is not administered systemically. In these local infections, the large numbers of organisms usually present, as well as substances such as those occurring in pus and necrotic tissue, reduce the activity of sulphonamides and, although the sensitivity of bacteria is probably increased to only a limited, variable extent by the Harper and Cawston technique, the use of lysed horse blood in culture media for tests of this kind may thus produce false results. The sulphacetamide sodium, however, may gain advantage in our investigations through light inoculation of the media and through the use of media containing 0·5 per cent. of "Lab-Lemco" and 1·0 per cent. of "Oxoid" peptone.

Summary and Conclusions

Although the species and strains of organisms used in our short study are limited, the following inferences appear to be justified:—

1. Sodium propionate inhibits growth of a wide range of bacteria and fungi responsible for local infections.
2. The activity of the drug is not markedly affected by the presence of whole horse blood.
3. The compound exhibits greater bacteriostatic and fungistatic effects in acid media than in alkaline media.
4. When tested *in vitro* in neutral media, sodium propionate appears to be rather less active than sulphacetamide sodium against staphylococci. This conclusion does not appear to be confirmed by unpublished results of clinical trials and *in vivo* investigations undertaken with the collaboration of veterinary practitioners; the variance may be due to factors such as difference in strains and the presence of sulphonamide antagonists in infected areas.
5. There is evidence that bacteria may develop resistance to sulphacetamide sodium more readily than is the case with sodium propionate, and this inference has been supported by therapeutic trial.
6. Tests with *Staphylococcus aureus* suggest that antagonism exists between sodium propionate and sulphacetamide sodium. There thus appears to be no justification for the use of a mixture of these drugs, especially since the addition of the sulphonamide would introduce the risk of sensitisation.

We desire to acknowledge the valuable assistance of Mr. S. J. Tucker.

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PROPIONATES OF SODIUM, POTASSIUM AND CALCIUM

Chemical names	Sodium propionate	Potassium propionate	Calcium propionate
Empirical formula	$C_3H_5O_2Na$	$C_3H_5O_2K$	$C_6H_{10}O_4Ca$
Structural formula	$CH_3 \cdot CH_2 \cdot COONa$	$CH_3 \cdot CH_2 \cdot COOK$	$(CH_3 \cdot CH_2 \cdot COO)_2Ca$
Molecular weight	96.06	112.17	186.23

Definition Not less than 99% in the dry state.

Description White or colourless, crystalline solids; soluble in water to about 30%

Natural occurrence Propionates occur naturally in fermented foods, as well as in human perspiration and in the products of rumen digestion.

Use As mould inhibitors in foodstuffs.

Biological Data

Acute toxicity

The LD_{50} of propionic acid administered orally to the rat was found to be 2600 mg/kg body weight.[1] The sodium and potassium salts were well tolerated.[2, 3] Solutions of propionates applied to the eye in concentrations up to 15% in man and up to 20% in rabbits had no irritating effect.[4]

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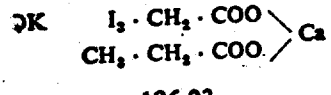
- IUM AND CALCIUM

Calcium propionate

$C_6H_{10}O_4Ca$



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rabbits had no irritating

Short-term studies

Rat

4 groups of 8 rats were kept for 4 weeks on diets containing 1% and 3% of sodium or calcium propionate. No effect on growth was observed.[5]

Man

In an adult male, daily oral doses of 6000 mg of sodium propionate rendered the urine faintly alkaline but had no other effect.[3]

Long-term studies

Rat

Sodium propionate fed to large numbers of rats in the diet at a concentration of 3.75% for one year had no effect on growth or mortality rate, nor any other toxicological effect as judged by the organ weights and histological findings in the organs studied.[6, 7]

Biochemical aspects

Propionates are metabolized and utilized in the same way as a normal fatty acid.[8] Isotopic sodium propionate fed to fasted rats gave rise to glucose in the liver.[9] In the mouse, ^{11}C-tagged propionate has been shown to act as a precursor of body fat,[10] and to be incorporated into the odd-numbered fatty acids of milk fat.[11] Furthermore, the labelled carbon has been found in CO_2, glucose, succinate, malate, fumarate and proteins.[12] Propionate combines with coenzyme A in the same way as acetic acid, becoming carboxylated to methyl-malonyl-coenzyme A and undergoing a quantitative conversion to succinate which then enters the Krebs cycle.[13] According to *in vitro* experiments, acetate metabolism may be inhibited by propionate. The production of $^{14}CO_2$ from labelled acetate by rat liver homogenates was almost abolished by propionate in only one tenth the concentration of the acetate.[14] Inhibition of catalase activity has been reported.[15]

Comments on experimental studies reported

There are no toxicological studies of longer duration than one year. *In vivo* studies bearing on the possibility of interference with acetate metabolism are lacking, but there is sufficient evidence that propionate is metabolized as a normal fatty acid.

Evaluation

Levels causing no significant toxicological effect in the rat

3.75% (= 37 500 p.p.m.) in the diet, equivalent to 2000 mg/kg body weight per day.

Estimate of acceptable daily intakes for man

	mg/kg body weight
Unconditional acceptance	0-10
Conditional acceptance	10-20

Further work considered desirable

Biochemical studies on interference of propionate with acetate metabolism *in vivo*.

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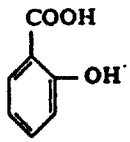
SALICYLIC ACID

Chemical names *o*-Hydroxybenzoic acid; *o*-oxybenzoic acid

Empirical formula $C_7H_6O_3$

Structural formula



Molecular weight 138.1

Definition Salicylic acid contains not less than 99.5% of $C_7H_6O_3$ after drying over sulfuric acid for 3 hours.

PROPIONIC ACID

Chemical names Propionic acid; Propanoic acid

Empirical formula $C_3H_6O_2$

Structural formula CH_3CH_2COOH

Molecular weight 74.08

Definition Propionic acid is a liquid with a slightly pungent odour.

Use As mould inhibitor

Biological Data

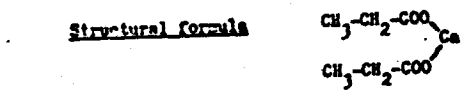
(See Sodium Propionate)

CALCIUM PROPIONATE

Chemical names Calcium propionate; Calcium propanoate

Empirical formula $C_6H_{10}O_4Ca$

Structural formula



Molecular weight 186.23

Definition Calcium propionate, after drying at 110°, contains not less than 99 per cent. of $C_6H_{10}O_4Ca$.

Description Calcium propionate is a white crystalline solid with not more than a faint odour of propionic acid.

Use As mould inhibitor.

Biological Data

(See Sodium Propionate)

SODIUM PROPIONATE

Chemical names Sodium propionate; Sodium propanoate

Empirical formula $C_3H_5O_2Na$

Structural formula $CH_3-CH_2-COONa$

Molecular weight 96.06

Definition Sodium propionate, after drying at 110°, contains not less than 99 per cent. of $C_3H_5O_2Na$.

Description Sodium propionate is a white or colourless, crystalline, hygroscopic solid with not more than a faint characteristic odour.

Use As mould inhibitor.

Biological data

Biological data on propionates are given in the Sixth Report of the Joint FAO/WHO Expert Committee on Food Additives, FAO Nutrition Meetings Report Series No. 31; Wld Hlth Org. techn. Rep. Ser., 229.

Evaluation

There is no reason to believe that propionic acid differs toxicologically from its calcium and sodium salts. The estimated acceptable daily intakes of these salts for man given in the Sixth Report of the Joint FAO/WHO Expert Committee on Food Additives are therefore considered applicable to the acid.

Estimate of acceptable daily intakes for man

	mg/kg body-weight[1]
Unconditional acceptance	0-10
Conditional acceptance	10-20

[1] As the sum of propionic acid and calcium and sodium propionate (calculated as propionic acid).

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... those of Nachlas *et al*(7), we obtain a value of 17.8 units. Barka and Dallner reported a mean of 7 mg formazan reduced per ... wet weight in 30 minutes(8). Our values for 30 minutes would be approximately 35 ... Kun *et al* reported 1.6 μg dye reduced per mg liver in 10 minutes(9). For this unit, our value would be approximately 12.4 μg. Our values are thus within the wide range as reported by others.

Summary. A micro method for quantitative determination of succinic dehydrogenase (SDH) activity was applied to frozen, vacuum-dried sweat gland. SDH activity determinations were made on cystic fibrosis children and controls. No significant difference between the two groups appeared.

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Stimulation of Gastric Acid Secretion by Acetate. (30214)

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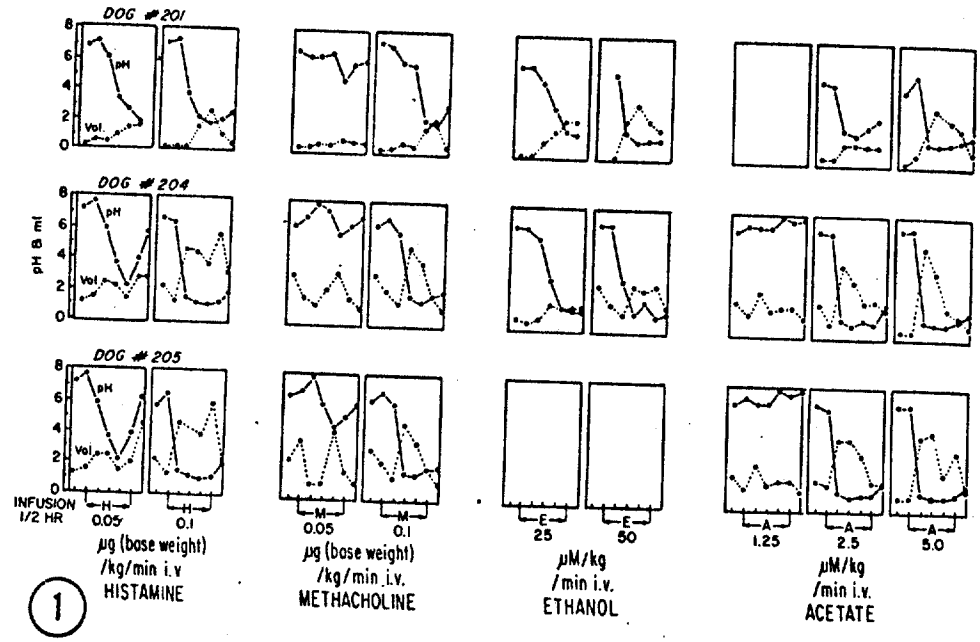
Although ethyl alcohol has long been known to stimulate gastric acid secretion, there is no agreement concerning the mode of action. Dragstedt *et al*(2) observed that perfusion of organs with dilute alcohol solution resulted in histamine-like activity in the perfusate. They considered histamine release to be a possible mechanism. However, Irvine, Watkin and Williams(6) did not detect a rise in free histamine concentration in the urine during alcohol stimulation but a histamine infusion producing equivalent acid response raised urinary histamine levels. Woodward, Slotten and Tillmanns(12) reported that alcohol applied directly to antral mucosa was a powerful stimulant for the antral gastrin mechanism. Irvine, Watkin and Williams(6), although confirming the gastrin releasing effect of ethanol, did not believe that gastrin was the major factor mediating the acid response to oral or intravenous alcohol. Hirschowitz *et al*(5) interpreted results of experiments with human subjects to indicate a central mechanism for alcohol stimulation of gastric secretion mediated through the vagus nerve.

The stimulatory effect of acetate, a metabolic product of ethanol, was reported by McManus(11). Gastric stimulation by acetate was used by Zawoiski, Braunschweig and Beyer(13) in experiments with steroids. Certain similarities between acetate and ethanol stimulation were observed. In this paper are presented in more detail some of these observations.

Methods. Six female Heidenhain gastric pouch dogs with total antrum resections were used in the experiments. The dogs ranged in weight from 10.0-12.7 kg. The animals were prepared surgically several months before they were used experimentally. Test meals determined the adequacy of resection of antrum. The animals had been trained to stand in a loose sling and were not anesthetized during experiments. Collections of gastric secretion were made into 12 ml graduated centrifuge tubes attached to stainless steel gastric cannulas. Acidity of collections was measured with a Beckman glass electrode pH meter. Since the small volumes of some of the collections limited the analysis, gastric acidity was expressed in terms of pH. The

gastric acid stimulants were administered intravenously by a constant velocity infusion pump at the rate of 2 ml/minute. Before stimulation, the control phase secretion was measured at ½-hour intervals for 1 hour. During a 2-hour period of intravenous in-



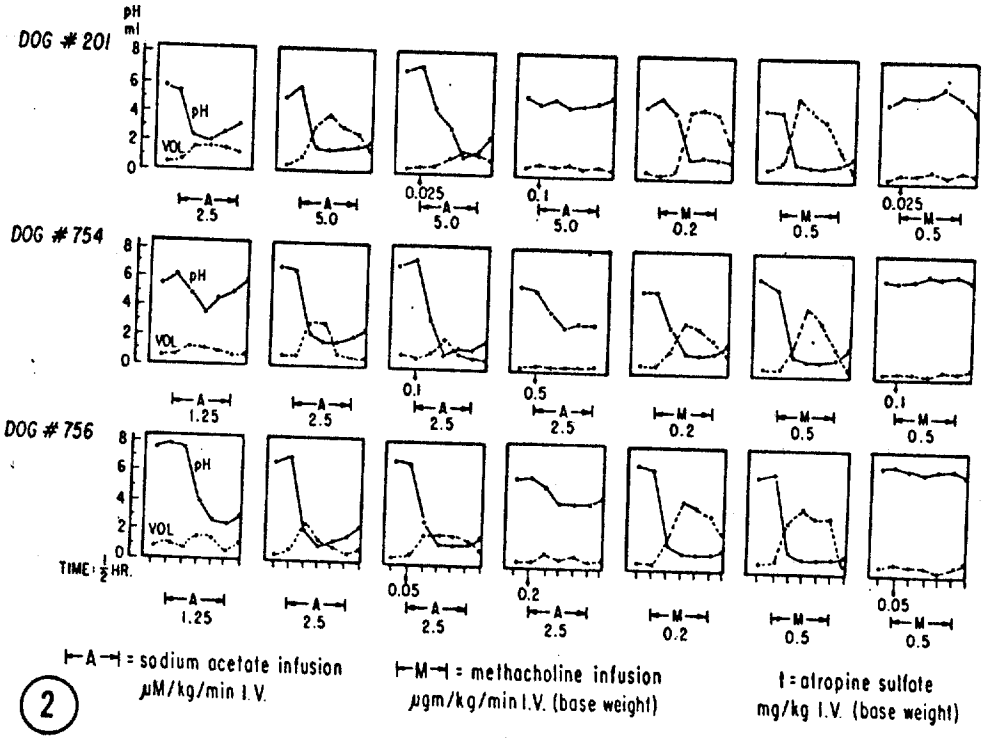


FIG. 1. Response of 3 dogs to stimulants of gastric acid secretion.
FIG. 2. Inhibitory effect of atropine.

fusion which followed immediately after the control phase, four ½-hour collections were made. An additional ½-hour sample was collected after termination of the infusion. Mett's method(4) was used for determination of pepsin.

Results. Infusion of sodium acetate in doses of 2.5-5.0 μM/kg/minute had an immediate pronounced stimulatory effect on secretion of acid. Volume rose sharply and the pH dropped to about 1. Data that compare the stimulatory effect of histamine, methacholine, ethanol and sodium acetate on three dogs are summarized in Fig. 1. Each plot is the average of results for 2 separate experiments.

A large number of acids were tested for similar action. Sodium propionate was approximately as potent as sodium acetate. β-Alanine and sodium hydracrylate(7,8) were moderately active in doses of 10 μM/kg/minute. The following sodium salts were inactive when infused in a dose of 50 μM/kg/minute: butyrate, pyruvate, lactate, oxalacetate, α ketoglutarate, fumarate, succinate, glutamate and acetoacetate.

The effects of atropine on secretion stimulated by methacholine and by sodium acetate were compared. In these experiments, the dose of methacholine was determined which produced equivalent or greater stimulation than the minimally effective doses of acetate. Then the inhibitory effect of atropine was determined. The doses of atropine required to suppress the effect of acetate were higher than those required to inhibit methacholine. Fig. 2 summarizes the results of the tests with atropine. The plots are averaged values for 2 experiments.

The peptic activity of secretion evoked by sodium acetate and by methacholine were compared. During a 1½-hour period of stimulation with sodium acetate, 5.0 μM/kg/minute, the peptic activity tended to decrease slightly. Methacholine, 0.2 μg/kg/minute, produced an equivalent secretion in respect to pH and volume but with very much higher peptic power. Results of experiments with 3 dogs are summarized in Fig. 3. One experiment was performed with dog #204. The data for dogs #756 and 705 are averaged values for 2 experiments with each animal.

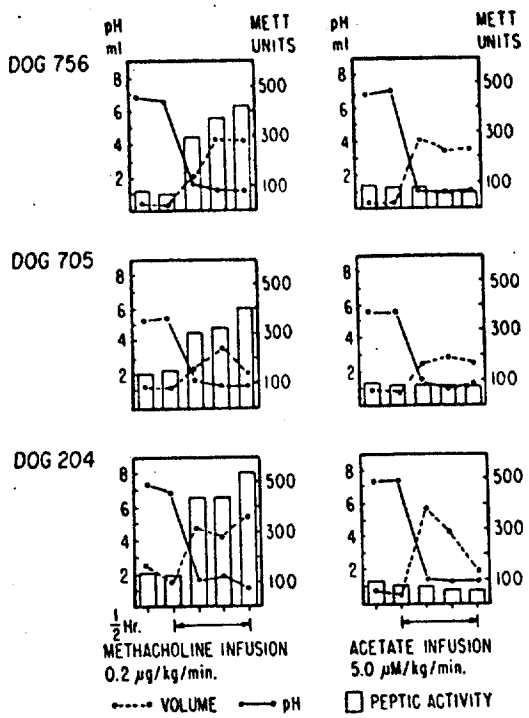


FIG. 3. Peptic activity of gastric secretion stimulated by acetate and methacholine.

Discussion. In regard to 2 parameters, acid secretion stimulated by acetate and ethanol were similar. Secretion stimulated by ethanol is known to be relatively low in peptic activity(9). This was also found for acetate stimulated secretion. Secondly, ethanol stimulation in the dog is readily inhibited by atropine but in a dose higher than required to inhibit secretion mediated by cholinergic stimulation(3). The acetate effect was likewise inhibited by atropine but in a dose higher than that required to block stimulation by methacholine.

The effect of infusion of acetate on plasma levels of the compound was not determined. Annison(1) reported normal plasma acetate levels of approximately 0.1 to 0.25 mM/l for the dog. For man, the levels were approximately 0.2 mM/l. Lundquist(10) using a different method, reported normal levels of about 0.05 to 0.1 mM/l for the human.

In view of the normal plasma levels for acetate cited above, the quantities of acetate required for stimulation of gastric secretion appear to be extremely low. This is all the

more reason to suspect that the results herein reported have significance from a physiological and biochemical standpoint.

Summary. Sodium acetate or propionate, administered by venoclysis in a dose of 2.5-5.0 μM/kg/minute, had a pronounced stimulatory effect on secretion of acid from the Heidenhain pouch of the dog with total antrum resection. Higher doses of atropine were required to suppress acetate than methacholine stimulation. Acetate-stimulated secretion was lower in peptic activity than secretion produced in response to methacholine. Similarities of acetate and ethanol stimulation were recognized and discussed.

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Movement of Calcium in Both Directions Across the Primate Placenta.* (30215)

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The continuous movement of a myriad of nutrient substances and metabolic waste products across the placenta from mother to fetus and in the reverse direction is vital to the growth and development of the embryo. Radioactive tracer techniques have been used for the study of placental transport of the essential nutrient, calcium, in mice, rats, rabbits and cattle(1-4). The rapid movement of strontium in both directions across the monkey placenta has been directly demonstrated in monkeys(5). This report concerns the simultaneous transfer of isotopically labeled calcium across the placenta from mother to fetus and in the reverse direction. The primary intent was to estimate the approximate magnitudes of these transfers. Pregnant monkeys were chosen because of the close similarity of their placental structure to that of humans(6,7).

Method. Each of 3 pregnant rhesus monkeys was anesthetized with intramuscular piperidine HCl (4 mg/kg supplemented with pentobarbital 10 mg/kg body weight). Polyethylene catheters were placed in a maternal femoral vein, an interplacental vessel of the fetus and the amniotic sac in a manner previously described(6). Five μc of I^{131} labeled human serum albumin in 2 ml of isotonic saline were injected into each fetal and maternal catheter, for estimation of blood vol-

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Canadian Journal Biochemistry 45(1) 1967

OBSERVATIONS ON SOME EFFECTS OF THE SODIUM SALTS OF CERTAIN MONOCARBOXYLIC ACIDS ON ESTABLISHED CELL LINES[1]

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Received July 8, 1966

There appears to be very little information in the literature pertaining to concentrations of various air pollutants or tobacco-smoke constituents that may be toxic or nontoxic to tissue cells cultivated in vitro. We have undertaken the task of ascertaining these levels and this report records observations concerning the first few of many known pollutants.

The effects of the sodium salts of 10 monocarboxylic acids (formate, acetate, propionate, butyrate, valerate, caproate, oenanthylate, caprylate, caprate, and benzoate) on several established cell lines (HeLa, strain L, human lung, human skin) were studied.

All of these compounds at a concentration of 10 mg % were toxic to the cell lines tested with the exceptions of formate, acetate, and benzoate on strain L, and of valerate and caprylate on human lung.

All the compounds either stimulated proliferation or had no significant effect at 1 mg % except caproate and benzoate, which were toxic to human lung and human skin cells, respectively.

Introduction

It has been recognized for some time that polluted air and tobacco smoke contain acidic components. Most earlier investigations on the occurrence of acids such as acetic, formic, and butyric (1), were based on qualitative determinations and only recently have quantitative studies been made. Buyske and colleagues (2) undertook a quantitative identification of the individual steam-volatile monocarboxylic acids composing 5–8% of the particulate phase of tobacco smoke. The following experiments are concerned with these acids.

Although there is little evidence of carcinogenic activity by any of these compounds (3, 4), they may be of biological and medical importance otherwise. Some of them, especially those with a carbon chain length of C_8 to C_{14}, exhibit rather pronounced antitumor activity (5–7).

Ciliastatic action of tobacco smoke has been reported by numerous investigators (8–13). According to Wynder *et al.* (13), the effect seems to be due to some extent to the acidic and phenolic fractions of smoke condensate. They found that the ciliatoxicity produced by formic acid, acetic acid, propionic acid, benzoic acid, and formaldehyde was more pronounced, and that produced by acetaldehyde, benzaldehyde, *n*-butyric acid, and oxalic acid was less pronounced, than the effects of phenol.

Some of the steam-volatile organic acids were chosen for these studies to obtain information regarding their toxicity in cells cultivated in vitro. This

[1]These investigations were supported by Grant No. AP00092-08, Division of Air Pollution, Bureau of State Services, Public Health Service and a grant from the Council for Tobacco Research, U.S.A.

additional information concerning some of the biological effects of these constituents of polluted air and tobacco smoke should prove to be of interest and value to other investigators.

Materials and Methods

Human skin cells (NCTC Clone 2414), strain L cells (NCTC Clone 929), and HeLa cells were used in testing some of the compounds, and human fetal lung cells were utilized in all the tests. The cells were cultured in NCTC 109 medium supplemented with 10% horse serum (14–16) to which was added, at desired concentrations, the compound under investigation.

Short-term experiments, extending to 19 days, were conducted to ascertain toxic and nontoxic concentrations.

Cultures were replicated in T-15 flasks by the usual procedures (17). Two milliliters of cell suspension were added to each flask. After an adjustment period of 48 hours the flasks were divided into control and experimental groups. Thereafter, the medium was changed every 48 hours and the cells in each of three flasks, selected at random from each group, were counted. Counts were made with a Coulter electronic counter.

Erythrosin B was used for testing viability (18). Microscopic examinations were made to ascertain morphological changes. In some instances photographs were taken to record any alterations observed.

Results and Discussion

The results of the experiments are presented in Table 1. Some explanation is necessary concerning the two terms "effect" and "morphological changes".

Several terms were used to describe the effects of a compound upon the cells. The effect was described as "toxic" if the cells died or if there was a decrease in their population. If proliferation occurred, but to less extent than that of the control cells, the term "retarding" was used. An effect resulting in a significantly greater population than that of the controls was considered "stimulating". "Negligible" denoted neither significant inhibitory nor stimulatory effects. The table does not indicate, however, the occasional instances when during the first 48 to 96 hours the compound appeared to stimulate growth, although often this period of stimulation was followed by a retarding effect.

Morphological alterations, observed under ordinary microscopic examination, were varied. One of the most prominent and frequent changes was a tendency of the cells to take on a spherical shape, and this was usually followed by detachment. An increase in number and size of vacuoles as well as in granule formation was not uncommon. Experimental cells sometimes appeared to be enlarged, and were frequently filamentous and spindle-shaped (at least, more so than control cells). Giant cells developed occasionally and in some instances blebs appeared on the surface. In the table, the terms used for morphological changes indicate only that changes were or were not observed, and are in no way descriptive. When the appearance was recognized as definitely altered, it is indicated in the table that the alteration took place within a certain

time or time range. If no change could be detected, the term "none" is used, although this is based only on observations made at a magnification of about 400 times. Fixation of the cells, which occurred at times when they were exposed to the higher concentrations of some compounds, was often difficult to recognize, since appearance was not appreciably altered and detachment did not necessarily occur. However, viability or indications of fixation could be ascertained by means of erythrosin B.

Observations were often made at 24-hour intervals and routinely made at least every 48 hours.

A concentration of 2000 mg % was used only with sodium formate and acetate. Sodium formate, at this concentration, appeared to be more toxic to strain L cells, which were dead within 24 hours, than to human skin cells, which continued to live for approximately 96 hours. Both types of cells displayed obvious changes after the first 24 hours. Sodium acetate at a concentration of 2000 mg % produced a declining population in human skin cells, but in strain L cells a retarding effect occurred, in comparison with controls. Again, both cell types showed morphological changes within 24 hours.

The results obtained when cells in vitro were subjected to a range of concentrations of the salts are presented in Table I. In concentrations of 1000 mg %, acetate resulted in either a retarded rate of proliferation or a declining population. Formate retarded proliferation of strain L cells and caused a progressively declining population of human skin cells.

It is interesting to note that all concentrations (10 mg % and above) of *formate* and *acetate* had a growth-retarding effect, except on strain L cells, and that the effect on HeLa cells appeared to be negligible. (The additional Na content at this and lower concentrations of the salts is negligible compared with the total Na content of the medium, e.g. the Na content in 10 mg % Na formate is about 5 mg %. The total Na content of the medium plus 10% serum was about 400 mg %.) Proliferation in strain L seemed to be enhanced when the cells were exposed to this concentration. Formate at 1 mg % also appeared to stimulate proliferation in human skin cells. Usually, if the so-called "retarding" effect was comparatively slight no morphological changes were observed.

Sodium *propionate* appeared to be toxic to human lung cells only in concentrations above 10 mg %. No other cell lines were tested.

Sodium *butyrate* in concentrations up to 1 mg % seemed to have very little if any effect on the three lines indicated. At 10 mg % and above it became quite toxic.

Sodium *valerate* at 10 mg % was toxic to all cell lines except human lung cells, on which there was very little effect, if any.

Only human lung cells were exposed to *caproate*, which was toxic, especially above 10 mg %.

The results indicate that *oenanthylate* at a concentration of 1 mg % had no effect on human lung cells but had a retarding effect and was quite toxic above 10 mg %.

TABLE I

The effects of various concentrations of the sodium salts of some monocarboxylic acids on several established cell lines (explanation of "Effect" and "Morphological changes" given in the text)

Substance tested	Cell line	Concentration of substance tested (mg %)	No. of cells/ml at exposure ($\times 10^6$)	Time to maximum population (hours after exposure)	Maximum population ($\times 10^6$)	Effect	Morphological changes
Sodium formate	Human skin (NCTC 2414)	0	0.152	144	0.411	—	—
		1	0.152	144	0.492	Stimulating (?)	None
		10	0.152	96	0.309	Retarding	None
		100	0.152	144	0.271	Retarding	Within 48 hours
		1000	0.152	—	—	Dead within 144 hours	Within 24 hours
	Strain L (NCTC 929)	0	0.132	144	1.084	—	—
		1	0.132	144	1.174	None	None
		10	0.132	144	1.640	Stimulating	None
		100	0.132	144	0.903	Retarding (?)	None
		1000	0.132	144	0.140	Retarding	Within 48 hours
	Human lung	0	0.279	192	0.872	—	—
		1	0.279	144	0.851	None	None
		10	0.279	144	0.635	Retarding	Within 144 hours
		100	0.279	144	0.509	Retarding	Within 96–120 hours
		1000	0.279	—	—	Dead within 48 hours	Within 24 hours
	HeLa	0	0.356	240	0.760	—	—
		1	0.356	144	0.792	Negligible	None
		10	0.356	168	0.702	Retarding (?)	None
		100	0.356	240	0.668	Retarding	Within 240 hours
		1000	0.356	—	—	Dead within 24 hours	Within 24 hours
Sodium acetate	Human skin (NCTC 2414)	0	0.578	216	2.10	—	—
		1	0.578	216	1.95	None	None
		10	0.578	216	1.82	Retarding	None
		100	0.578	144	0.351	Retarding	None
		1000	0.578	—	—	Toxic	Slight
	Strain L (NCTC 929)	0	0.132	144	1.084	—	—
		1	0.132	144	1.087	None	None
		10	0.132	144	1.379	Stimulating	None
		100	0.132	120	0.666	Retarding	None
		1000	0.132	96	0.254	Retarding	Negligible

TABLE I (*Continued*)

TABLE I (*Continued*)

Substance tested	Cell line	Concentration of substance tested (mg %)	No. of cells/ml at exposure ($\times 10^6$)	Time to maximum population (hours after exposure)	Maximum population ($\times 10^6$)	Effect	Morphological changes
	Human lung	0	0.284	192	0.830	—	—
		1	0.284	216	0.792	None	None
		10	0.284	192	0.710	Retarding	None
		100	0.284	96	0.531	Retarding	Within 48 hours
		1000	0.284	—	—	Toxic	Within 12–24 hours
	HeLa	0	0.142	192	0.516	—	—
		1	0.142	192	0.510	None	None
		10	0.142	192	0.485	Retarding	Slight
		100	0.142	144	0.458	Retarding	Slight
		1000	0.142	192	0.292	Retarding	Within 144 hours
Sodium propionate	Human lung	0	0.310	144	1.060	—	—
		1	0.310	196	1.318	Stimulating	None
		10	0.310	196	1.149	None	None
		100	0.310	144	0.399	Retarding	Within 96 hours
		1000	0.310	—	—	Dead within 48 hours	Within 24 hours
Sodium butyrate	Human skin (NCTC 2414)	0	0.578	216	2.10	—	—
		1	0.578	192	1.952	None	None
		10	0.578	192	1.222	Dead within 144 hours	Within 48 hours
		100	0.578	—	—	Dead within 48 hours	Within 48 hours
		1000	0.578	—	—	Dead (24–48 hours)	Within 24 hours
	Strain L (NCTC 929)	0	0.132	144	1.380	—	—
		1	0.132	144	1.290	None	None
		10	0.132	48	0.490	Retarding	Within 48 hours
		100	0.132	—	—	Dead within 48 hours	Within 24–48 hours
		1000	0.132	—	—	Dead (24–48 hours)	Within 24 hours
	Human lung	0	0.272	144	0.840	—	—
		1	0.272	144	0.793	None	None
		10	0.272	96	0.462	Retarding	Within 96–120 hours
		100	0.272	—	—	Dead within 48 hours	Within 24–48 hours
		1000	0.272	—	—	Dead within 48 hours	Within 24 hours

TABLE I (*Continued*)

Substance tested	Cell line	Concentration of substance tested (mg %)	No. of cells/ml at exposure ($\times 10^6$)	Time to maximum population (hours after exposure)	Maximum population ($\times 10^6$)	Effect	Morphological changes
Sodium valerate	Human skin (NCTC 2414)	0	0.172	144	0.714	—	—
		1	0.172	192	0.682	None	None
		10	0.172	192	0.324	Retarding	Within 24 hours
		100	0.172	—	—	Dead within 96 hours	Within 24 hours
		1000	0.172	—	—	Dead within 24 hours	Within 24 hours
	Strain L (NCTC 929)	0	0.126	144	1.380	—	—
		1	0.126	144	1.300	None	None
		10	0.126	144	0.632	Retarding	Within 48 hours
		100	0.126	144	0.300	Retarding	Within 24–48 hours
		1000	0.126	—	—	Dead within 48 hours	Within 24 hours
	Human lung	0	0.281	192	1.104	—	—
		1	0.281	192	1.150	None	None
		10	0.281	192	1.090	None	Within 96 hours
		100	0.281	—	—	Toxic	Within 72 hours
		500	0.281	—	—	Toxic	Within 24 hours
Sodium caproate (hexanoic Na salt)	Human lung	0	0.344	144	0.990	—	—
		1	0.344	168	0.682	Retarding	Within 120 hours
		10	0.344	168	0.515	Retarding	Within 120 hours
		100	0.344	—	—	Dead within 96 hours	Within 24 hours
		500	0.344	—	—	Dead within 96 hours	Within 24 hours
Sodium œnanthylate (heptanoic Na salt)	Human lung	0	0.225	96	0.491	—	—
		1	0.225	96	0.480	None	None
		10	0.225	96	0.346	Retarding	Within 96 hours
		100	0.225	—	—	Dead within 48 hours	Within 24–48 hours
		500	0.225	—	—	Dead within 24 hours	Within 4 hours
Sodium caprylate (octanoic Na salt)	Human lung	0	0.310	144	0.660	—	—
		1	0.310	144	0.737	Stimulating (?)	None
		10	0.310	96	0.451	Retarding	Within 96–120 hours
		100	0.310	—	—	Dead (48–96 hours)	Within 24 hours
		500	0.310	—	—	Dead (24–48 hours)	Within 24 hours

TABLE I (*Concluded*)

TABLE 1 (*Concluded*)

Substance tested	Cell line	Concentration of substance tested (mg %)	No. of cells/ml at exposure ($\times 10^6$)	Time to maximum population (hours after exposure)	Maximum population ($\times 10^6$)	Effect	Morphological changes
Sodium caprate (decanoic Na salt)	Human skin (NCTC 2414)	0	0.156	216	0.710	—	—
		1	0.156	216	0.955	Stimulating	None
		10	0.156	144	0.233	Retarding	Within 96 hours
		100	0.156	—	—	Dead within 48 hours	Within 24 hours
		1000	0.156	—	—	Dead (24–48 hours)	Within 24 hours
	Strain L (NCTC 929)	0	0.175	144	1.255	—	—
		1	0.175	144	1.182	None	None
		10	0.175	120	0.355	Retarding	Within 48 hours
		100	0.175	—	—	Dead within 48 hours	Within 24 hours
		1000	0.175	—	—	Dead within 24 hours	—
	Human lung	0	0.424	144	1.110	—	—
		1	0.424	144	0.976	None	None
		10	0.424	120	0.276	Retarding	Within 72 hours
		50	0.424	—	—	Dead within 96 hours	Within 24–48 hours
		100	0.424	—	—	Dead (48–72 hours)	Within 24 hours
Sodium benzoate	Human skin (NCTC 2414)	0	0.156	216	0.710	—	—
		1	0.156	216	0.650	Retarding (?)	None
		10	0.156	144	0.517	Retarding	None
		100	0.156	144	0.434	Retarding	Within 120 hours
		1000	0.156	—	—	Dead (24–48 hours)	Within 12–24 hours
	Strain L (NCTC 929)	0	0.175	144	1.255	—	—
		1	0.175	144	1.306	None	None
		10	0.175	144	1.150	None	None
		100	0.175	120	0.805	Retarding	Within 96 hours
		1000	0.175	—	—	Dead (48–96 hours)	Within 12–24 hours
	Human lung	0	0.189	168	1.146	—	—
		1	0.189	168	1.070	None	None
		10	0.189	144	0.474	Retarding	Within 48–72 hours
		100	0.189	120	0.373	Retarding	Within 48–72 hours
		1000	0.189	—	—	Dead within 72 hours	Within 24 hours

Caprylate at 1 mg % actually enhanced proliferation of human lung cells but was toxic at 10 mg % and above.

Sodium *caprate* stimulated proliferation of human lung cells but appeared to have no effect in concentrations of 1 mg %. Greater concentrations were toxic in all cases.

Sodium *benzoate* at 1 mg % appeared to have very little effect on any of the three lines tested, but except for strain L it was toxic above this concentration.

Aromatic polycyclic hydrocarbons, some of which are carcinogenic, may be adsorbed by soot particles in polluted air (19–22). These adsorbed carcinogenic hydrocarbons are considered biologically inactive (22, 23). Organic acids such as those occurring in tobacco smoke and polluted air may act as solvents for these carcinogenic hydrocarbons. Therefore, if soot and tobacco smoke should occur simultaneously in the lungs, the carcinogens possibly might be eluted from the soot by the organic acids of the smoke. The eluted carcinogens might then become biologically active (22).

Smog and tobacco smoke are known to be irritating to the mucous epithelium of the respiratory tract. This irritant nature may be due in part to acids and could facilitate further action, especially on the basal cells, by carcinogens.

Our interest in toxicity levels for the numerous air pollutants and tobacco-smoke constituents is based on our intention to investigate further their actual effects on basic mechanisms within cells. Additional information concerning biological effects of these constituents should prove to be of interest and value to other investigators.

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Translator's note: In the reproduction submitted for translation, one margin is badly blurred and sometimes illegible.

Arch. Sci. biol., 1955, 39, pp. 304-307 (Italian)

The Biologic Activity of Some Acids, Ketones and Aliphatic Esters (*)

By C. Romano

From the Institute of Legal and Insurance Medicine of the University of Naples

Foreword

The present publication continues a group of research projects on the pharmaco-dynamics of metabolites of alcohol (acetaldehyde, acetoin) and accompanying products (aldehydes in C_2, C_3, C_4; acids in C_3, C_4, C_6, C_7; acetoin, methylethyl ketone, diacetyl, ethyl acetate) for the purpose of defining a role they may have in the pathology of ethylism.

The effects of the aldehydes have already been intensively investigated (Romano and Meyers, 1; Romano, Meyers and Anderson, 2), also in relation to the antabuse-alcohol reaction and to alcoholic deaths due to circulatory collapse; there will now be reported the effects of the above specified acids, ketones and esters on blood pressure, respiration, and isolated organs.

That research on alcohol is of interest in legal medicine is documented by the publications dealing with the topic (cf. Palmieri, 3, pp. 8??-943), while the economic-social interest emerges from the consideration that the wind industry is one of the main resources of the Italian economy.

Experimental Part

I) Propionic, butyric, caproic and heptylic acids

It was felt to be important to establish for them a level of activity, since some previous publications had shown them to have a characteristic pharmacological action, despite their rapid oxidizability. Hall and Waldman

(4) have, in fact, pointed out that the fatty acids in C_6, C_7, C_8, C_{10} could induce ventricular arrhythmia. They injected the sodium salt of the acids, but in a vehicle of 3 ml.. of concentrated serum albumin.

Technique. The acids were converted into their water-soluble sodium salts (and) the solutions had a pH of 7.7 on the Beckman potentiometer. The effects on the pressure were studied in cats anesthesized with sodium pentobarbital, recording at the carotid with a mercury manometer. The doses used, intravenously, were: Sodium propionate (mol.wt. 74) 1×10^{-4} and 2×10^{-4} M/kg, sodium butyrate (mol. wt. 88) ... 10^{-4} M/kg, sodium caproate (mol.wt. 116) 1×10^{-4} M/kg, sodium heptylate (mol.wt. 130) 1×10^{-4} M/kg.

The effects on isolated organs were studied on rat uterus, by the method of Jalon, Bayo and Jalon (5), modified by Gaddum and Lembeck (6); the above sodium salts were added to the bath so as to obtain a final concentration of ... 10^{-6}, 5×10^{-6}, 1×10^{-5} M/ml.

Results. Effects on pressure were noted only after administration of sodium propionate, which proves to be hypotensive. The higher dose leads to a pressure decrease of about 40 mm Hg for the duration of 1 min; the smaller dose causes a hypotension of about one half. Cases of arrhythmia were not noted. Sodium heptylate at the dose of 20 mg/kg, i.v., was found to be inactive also in the dog.

Fig. 1. Rat uterus after Jalon et al. (5). At A, sodium heptylate is added at the dose of 1×10^{-5} M/ml; at B, adrenalin at the dose of 0.002 mcg/ml. The arrows indicate the effects.

Effects on rat uterus, on the other hand, were observed only in the case of this latter salt which, in the stronger concentration used, inhibited the contractions induced by carbacholine in a manner equivalent to a concentration of adrenalin in the bath of 0.002 mcg/ml. See fig. 1.

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II) Acetoin, methylethyl ketone and diacetyl

Westerfeld and Berg (7) have shown that acetoin normally occurs in the blood and in the urine, that it can be produced by the anion pyruvate + acetaldehyde by enzymatic condensation, that it is probably an intermediate substance in the metabolism of alcohol, and that when injected, it is metabolized without giving rise to hepatic glycogen. Besides, acetoin is more toxic than alcohol with additive toxicity, but less toxic than acetaldehyde, and it has in rats a LD_{50} of 2 g given intraperitoneally (Greenberg, 8).

Technique. The effects on arterial pressure, recorded at the carotid with a mercury manometer, and on respiration, were studied in dogs anesthesized with sodium pentobarbital. The ketones were used in awueous solution at the dose of 10 and 20 mg/kg, i.v.

The effects on isolated organs were studied on the rat uterus (cf. above) for acetoin only (mol.wt. 88) which had a final concentration in the bath of 5×10^{-6} and 1×10^{-5} M/ml.

The anticonvulsivating properties were studied in white mice subjected to metrazol shock.

Results. Only acetoin produces, at the higher dose, a slight pressure reduction and a certain polypnea; also on rat uterus it showed an activity inhibiting the contractions induced by carbacholine (fig. 2; 5×10^{-6} M/ml of acetoin were equivalent to 0.004 mcg/ml of adrenalin). It exhibited, moreover, a certain protective anticonvulsivating action.

Fig. 2. Rat uterus after Jalon et al. (5). At A, acetoin is added at the dose of 5×10^{-6} M/ml; at B, adrenalin at the dose of 0.004 mcg/ml. The arrows indicate the effects.

III) Ethyl acetate

It is known that the esters are less toxic than the acids and alcohols

from which they derive. To anesthesize a cat, for example, there are needed 43 mg/l of ethyl acetate in the ambient air.

Technique. The effects of ethyl acetate, used in aqueous solution, at the doses of 10 and 20 mg/kg, i.v., on the pressure and respiration, were studied on dogs anesthesized with sodium pentobarbital. The pressure was taken at the carotid with a mercury manometer.

Results. The higher dose of ester produced a slight hypotension for a short time and a certain degree of polypnea.

Conclusions

From the aggregate of the data set forth, the following appears.

a) The acids, ketones and esters examined, at the concentrations at which they are generally present in alcoholic beverages, should not by themselves have any characteristic effects.

b) The propionate anion is the most active on the pressure, while acetoin does not have on the circulation the qualitative and quantitative effects which the other metabolite of alcohol, acetaldehyde, causes under certain conditions, at equivalent doses (1, 2).

c) The very weak adrenalin-like activity of the fatty acids in the preparation of Jalon et al. (5), believed to be one of the most specific biologic tests for epinephrine, suggests that the ventricular arrhythmias described (4) are attributable to mechanisms different from those occurring in adrenalin intoxication.

d) Acetoin, at equimolarity of doses, is the strongest inhibitor of the rat uterus.

Summary. The fatty acids in C3, C_4, C_6, C_7, acetoin, methylethyl ketone, diacetyl, ethyl acetate have weak pharmacological actions, and therefore, at the concentrations at which they are present in alcoholic beverages, cannot be involved in the pathology of ethylism.

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(*) The respective experiments were started by Dr. F. H. Meyers in the Department of Pharmacology of the University of California and were completed at the Istituto di Medicina legale of the University of Naples.

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Arch. Sci. biol., 1955, 39, 301-307.

L'ATTIVITÀ BIOLOGICA DI ALCUNI ACIDI, CHETONI E ESTERI ALIFATICI *).

Di C. Romano.

Dall'Istituto di Medicina legale e delle Assicurazioni della Università di Napoli

Premesse

L'attuale pubblicazione continua un gruppo di ricerche dedicate alla farmacodinamica di metaboliti dell'alcool (acetaldeide, acetone) e di prodotti che si accompagnano a esso (aldeidi in C_2, C_3, C_4; acidi in C_3, C_4, C_6, C_7; acetoino, metiletilchetone, diacetile, acetato di etile) allo scopo di precisarne un eventuale ruolo nella patologia dell'etilismo.

Gli effetti delle aldeidi sono stati già intensivamente investigati (Romano e Meyers, 1; Romano, Meyers e Anderson, 2), in rapporto anche alla reazione antabuse-alcool e alle morti alcooliche da collasso circolatorio; ora vengono riferiti gli effetti degli acidi, chetoni ed esteri prima specificati, sulla pressione, respirazione e organi isolati.

L'interesse medico-legale di ricerche sull'alcool è documentato dalle pubblicazioni dedicate all'argomento (cfr. Palmieri, 3, pp. [illegible] 943), mentre quello economico-sociale emerge dalla considerazione che l'industria dei vini è una delle principali risorse dell'economia italiana.

Parte sperimentale

I) *Acidi propionico, butirrico, capronico ed eptilico*

È stato ritenuto importante stabilire per essi un livello di attività dato che alcune precedenti pubblicazioni ne avrebbero dimostrato un'azione farmacologica caratteristica, nonostante la loro rapida ossidabilità. Hall e Waldman (4) segnalarono, infatti, che gli acidi grassi in C_6, C_7, C_8, C_{10} potevano indurre aritmie ventricolari. Essi iniettarono il sale sodico degli acidi, ma in un veicolo di 5 g% di albumina serica concentrata.

*) Gli esperimenti relativi furono iniziati col Dott. F. H. Meyers nel Dipartimento di Farmacologia della Università di California e sono stati integrati e condotti a termine nell'Istituto di Medicina legale della Università di Napoli.

Tecnica. Gli acidi furono convertiti nei loro sali sodici solubili in acqua soluzioni avevano un pH di 7,7 al potenziometro di Beckman. Gli effetti sulla pressione furono studiati in Gatti anestetizzati con pentobarbital sodico, registrando carotide con manometro a mercurio. Le dosi usate, per via intravenosa, furono: propionato sodico (p. m. 74) 1×10^{-4} e 2×10^{-4} M/kg, butirrato sodico (p. m. 88) 10^{-4} M/kg, caproato sodico (p. m. 116) 1×10^{-4} M/kg, eptilato sodico (p. m. 130) 1×10^{-4} M/kg.

Gli effetti su organi isolati furono studiati sull'utero di Ratto, col metodo di Bayo e Jalon (5), modificato da Gaddum e Lembeck (6); i sali sodici suddetti furono aggiunti al bagno in modo da aversi una concentrazione finale di 10^{-6}, 5×10^{-6}, 1×10^{-5} M/ml.

Risultati. Effetti sulla pressione furono notati solo dopo somministrazione di propionato sodico, il quale riesce ipotensivo. La dose maggiore induce una caduta pressoria di circa 40 mm di Hg della durata di 1 min; la dose minore determina un'ipotensione di circa la metà. Non furono notate aritmie. L'eptilato sodico alla dose 20 mg/kg, i. v., si è rivelato inattivo anche nel Cane.

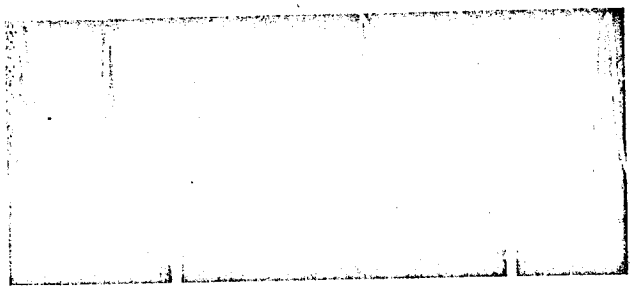

Fig. 1. - Utero di Ratto secondo Jalon *et alii* (5). In A viene aggiunto eptilato sodico nella dose di 1×10^{-5} M/ml; in B adrenalina nella dose di 0,002 μg/ml. Le frecce indicano gli effetti.

Effetti sull'utero di Ratto, viceversa, furono osservati nel solo caso di quest'ultimo sale, il quale, nella più forte concentrazione usata, inibì le contrazioni indotte dalla carbacolina in modo equivalente a una concentrazione di adrenalina nel bagno di 0,002 μg/ml. Si veda la fig. 1.

II) *Acetoino, metiletilchetone e diacetile*

Westerfeld e Berg (7) hanno dimostrato che l'acetoino occorre normalmente nel sangue e nelle orine, che può essere prodotto dalla reazione piruvato + acetaldeide per condensazione enzimatica, che è probabilmente un intermedio nel metabolismo dell'alcool e che ingerito è metabolizzato senza dar luogo a glicogeno epatico. Inoltre l'acetoino è più tossico dell'alcool con tossicità additiva, ma lo è

meno dell'acetaldeide e ha nei Ratti una DL_{50} di g 2 per via intraperitoneale (Greenberg, 8).

Tecnica. Gli effetti sulla pressione arteriosa, registrata alla carotide con manometro a mercurio, e sulla respirazione furono studiati in Cani anestetizzati con pentobarbital sodico. I chetoni furono impiegati in soluzione acquosa alle dosi di 10 e 20 mg/kg, i. v.

Gli effetti su organi isolati furono studiati sull'utero di Ratto (cfr. sopra) per il solo acetoino (p. m. 88) che aveva nel bagno una concentrazione finale di 5×10^{-6} e 1×10^{-5} M/ml.

Le proprietà anticonvulsivanti furono studiate in Topi bianchi sottoposti a *metrazol-shock*.

Risultati. Il solo acetoino produsse, alla dose maggiore, una lieve caduta pressoria e una certa polipnea; così pure sull'utero di Ratto rivelò un'attività inibitrice delle contrazioni indotte dalla

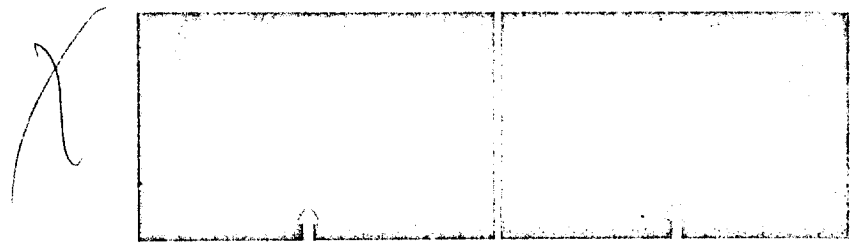

Fig. 2. - Utero di Ratto secondo Jalon *et alii* (5). In A viene aggiunto acetoino nella dose di 5×10^{-6} M/ml; in B adrenalina nella dose di 0,004 μg/ml. Le frecce indicano gli effetti.

carbacolina (fig. 2: 5×10^{-6} M/ml di acetoino furono equivalenti a 0,004 μg/ml di adrenalina). Esibì, inoltre, una certa azione protettiva anticonvulsivante.

III) *Acetato di etile*

È noto che gli esteri sono meno tossici degli acidi e degli alcooli donde derivano: per esempio per anestetizzare un Gatto occorrono 43 mg/l di acetato di etile nell'aria ambiente.

Tecnica. Gli effetti dell'acetato di etile, impiegato in soluzione acquosa, alle dosi di 10 e 20 mg/kg, i. v., sulla pressione e sulla respirazione, furono studiati su Cani anestetizzati con pentobarbital sodico. La pressione fu presa alla carotide con manometro a mercurio.

Risultati. La dose più elevata di estere produsse una lieve ipotensione per breve tempo e un certo grado di polipnea.

Conclusioni

Dall'insieme dei dati esposti, si rileva quanto segue.

a) Gli acidi, chetoni ed esteri esaminati, alle concentrazioni in cui sono generalmente presenti nelle bevande alcooliche, non dovrebbero avere effetti caratteristici di per sé.

b) Il più attivo sulla pressione è l'anione propionato, mentre l'acetoino non ha sul circolo gli effetti qualitativi e quantitativi che in certe condizioni determina, per dosi equivalenti, l'altro metabolita dell'alcool, l'acetaldeide (1, 2).

c) La debolissima attività adrenalino-simile degli acidi grassi nella preparazione di Jalon *et alii* (5), ritenuta uno dei *tests* biologici più specifici per l'epinefrina, dovrebbe far riferire le aritmie ventricolari descritte (4) a meccanismi diversi da quelli occorrenti nell'intossicazione adrenalinica.

d) L'acetoino, a equimolarità di dosi, è l'inibente più forte dell'utero di Ratto.

Riassunto. Gli acidi grassi in C_3, C_4, C_6, C_7, l'acetoino, il metiletilchetone, il diacetile, l'acetato di etile hanno deboli azioni farmacologiche e pertanto alle concentrazioni in cui sono presenti nelle bevande alcooliche non possono interferire nella patologia dell'etilismo.

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EFFECT OF FEEDING SODIUM PROPIONATE ON MILK AND FAT PRODUCTION, ROUGHAGE CONSUMPTION, BLOOD SUGAR, AND BLOOD KETONES OF DAIRY COWS[1]

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SUMMARY

A double-reversal experiment involving ten cows from the Cornell University Dairy Herd was used to detect the effect of the daily feeding of ½ lb. of sodium propionate on milk production, butterfat test, hay consumption, blood sugar and blood ketone levels. There were no significant differences ($P < 0.05$) in the milk production, hay consumption, blood sugar levels, blood ketone levels, or weight gains or losses attributed to the feeding of sodium propionate. There was a slight but nonsignificant fall in butterfat test.

In a supplemental experiment, two fistulated steers were fed propionate and control feed, and the fatty acid content of the rumen was determined at regular intervals after feeding. The feeding of the propionate feed resulted in higher levels of all the volatile fatty acids in the rumen for the first 3 hr. after feeding, particularly the propionic. From 3 to 9 hr. after feeding, the concentrations of rumen acids from the two feeds were quite similar. It was calculated that all of the administered propionate had disappeared in 7 hr., and most of it in 3 hr.

Sodium propionate has been shown to be effective in the treatment and prevention of ketosis. Schultz *(9)* found that the oral administration of ½ lb. of sodium propionate daily for from three to ten days to cows with uncomplicated ketosis was effective. Schultz *(10)* also found that the daily feeding of ¼ lb. of sodium propionate to cows for 6 wk. after calving reduced the incidence of ketosis. The propionate-fed cows showed higher blood sugar levels, lower blood ketone levels, and produced more milk for the first 6 wk. after calving than did the control cows.

Because of the stimulation in production observed when sodium propionate was fed or administered to cows with mild to severe ketosis, it was felt desirable to determine the effect of feeding it to normal cows. Information on this point was requested by those associated with official testing programs of the breed associations.

Tyznik and Allen *(11)* found that cows having a low butterfat test due to a high concentrate–low roughage diet showed a decreased percentage of acetic acid in the rumen, an increased percentage of propionic acid, and a normal percentage of butyric acid. When acetate was fed to these cows, the butterfat tests returned to normal. Miller and Allen *(7)* reported that the daily feeding of 1 lb. of sodium acetate to normal cows did not affect significantly the milk production, fat test, blood sugar or ketone values.

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[1] The data published in this paper are from a thesis presented by the senior author to the Graduate School, Cornell University, in partial fulfillment of the requirements for the degree of Master of Science, February, 1956.

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This experiment was designed to determine whether the feeding of sodium propionate would affect the milk production, fat test, roughage consumption, blood sugar and ketone levels of normal cows.

EXPERIMENTAL PROCEDURE

Eight Holstein and two Brown Swiss cows from the Cornell University Dairy Herd were used in this study. These cows were paired according to breed, age, and previous production. The cows were placed on the experiment on the 2nd wk. after calving. The 1st wk. after calving served as a preliminary period.

A double-reversal experiment was used, consisting of six experimental periods. Each period consisted of two weeks, with no transitional period between the experimental periods. Two types of grain were fed during the experimental periods. The first type was an 18% protein pelleted grain mix containing 5% sodium propionate. Poundages of composition were as follows: linseed oil meal and soybean oil meal, 200 each; gluten feed, distillers grains, and sodium propionate, 100 each; wheat bran, 300; ground oats, 500; hominy, 450; salt, 20; calcite, 10; dicalcium phosphate, 15, and trace minerals, 5. The other type of grain was the same mixture, except that the propionate was replaced by hominy feed. This served as the control feed.

One member of each pair was started on the control feed and the other member on the propionate feed. At the end of 2 wk. the type of grain was changed, and this was repeated every 2 wk. until a cow had been on three experimental periods of propionate feed and three periods of control feed.

Each cow received 10 lb. of propionate or control feed daily. During the propionate periods each cow received ½ lb. of sodium propionate from these 10 lb. of grain. The amount of grain required was determined from Appendix Table VIIIa in Morrison *(8)*. The difference between the 10 lb. of the basal grain mix fed and the total amount of grain required daily was made up of the regular herd mix containing 16% protein. The composition of this in pounds was as follows: ground oats, 460; ground corn or hominy, 400; wheat bran, 300; corn gluten feed, 500; soybean oil meal, 200; cane molasses, 100; iodized salt and steamed bone meal, 20 each; also, there was 2 g. of cobalt salt.

The amount of grain fed was changed at 4-wk. intervals, to insure that the same amount of grain was fed during one period of propionate feeding and one control period. An upper limit of 20 lb. of grain daily was set for all cows, to insure complete grain consumption. Each cow received 36 lb. of corn silage daily, and a mixed hay containing approximately 30% legume was fed ad libitum. Daily weigh-backs were taken on all feed, to insure accurate records of feed consumption.

Daily milk weights were taken and weekly composite milk samples were analyzed for butterfat content by the Babcock method. Blood samples were taken from the jugular vein at weekly intervals.

Potassium oxalate was used to prevent coagulation of the blood. The proteins were precipitated by the method described by Folin and Wu *(5)* and the blood sugar was determined colorimetrically by the method of Benedict *(2)*. Blood ketones were distilled from the filtrate by the method of Behre and Benedict *(1)* and the ketone bodies were determined as acetone by the method of Block and Bolling *(3)*.

Three-day weights were taken on all cows before they started on the experiment and before they were switched from one feed to another.

In a supplemental experiment, two dairy steers with rumen fistulas were used to determine the rate of disappearance of the propionic acid from the rumen. The steers were fed 5 lb. of control feed and 5 lb. of hay twice daily for 1 wk. At the end of a week, the fatty acids in the rumen were determined. A rumen sample was taken in the morning before the animal was fed and at hourly intervals after feeding for 10 hr. The 2nd wk., the steers received the same amount of propionate feed and hay, and at the end of the 2nd wk. the fatty acids in the rumen were determined by the same method.

The rumen samples were processed, distilled, and evaporated by the method described by Card *(1)*. The volatile fatty acids were separated according to the procedure of Marvel and Rands *(6)*, as modified by Card *(4)*.

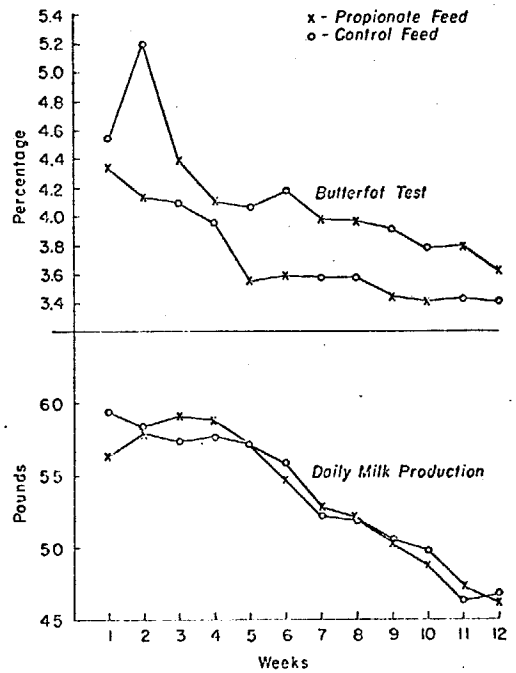

FIG. 1. Effect of feeding ½ lb. of sodium propionate daily during alternate 2-wk. periods on milk production and butterfat test.

RESULTS AND DISCUSSION

The average daily milk production by weeks is given (Figure 1). The daily feeding of ½ lb. of sodium propionate did not affect the milk production. The cows produced an average of 53.6 lb. of milk daily on the control feed and 53.5 lb. on the propionate feed. These results indicate that the stimulation in milk production observed following the feeding of propionate to ketosis cows was a result of a return to normal rather than a stimulation above normal.

A slight reduction in the butterfat tests occurred on the propionate feed. The weekly butterfat tests are also given (Figure 1). The feeding of propionate caused an average reduction of 0.11% in the butterfat tests during the entire experiment (Table 1). Analysis by the "t" test showed that this was not statistically significant. The high peak in the butterfat test during the 2nd wk. of control feeding was the result of two cows having exceptionally high butterfat tests. The butterfat tests for the first 6 wk. were more variable than for the last 6 wk.

TABLE 1

Summary of the effects of feeding one-half lb. of sodium propionate daily to dairy cows during alternate 2-wk. periods after calving

Measurement	Average values* for three 2-week periods on: Control feed	Propionate feed
Milk production *(lb/cow/day)*	53.6	53.5
Milk fat test *(%)*	3.98	3.87
Total fat *(lb/cow/period)*	30.1	29.1
Hay intake *(lb/cow/day)*	14.6	14.6
Silage intake *(lb/cow/day)*	35.0	35.3
Grain intake *(lb/cow/day)*	17.3	17.3
Blood glucose *(mg. %)*	50.0	50.7
Blood ketones *(mg. %)*	5.8	4.8
Body weight changes *(lb/cow/period)*	+0.1	−0.8

* Differences due to propionate were not statistically significant in the case of any of the measurements.

The effect of propionate feeding on blood sugar and blood ketone levels is shown (Figure 2). The general conclusion that can be drawn is that when the blood glucose and ketone values were normal, propionate feeding caused no appreciable change. However, when these values varied from the normal in the direction of ketosis, propionate feeding tended to cause them to return to normal. During the first 2-wk. period, the cows started on the control feed showed above-normal blood ketone levels and slightly low blood sugar values. The milk fat test was also unusually high (Figure 1). The feeding of propionate during the next 2-wk. period appeared to bring all of these measurements back toward normal. During the rest of the experiment, the blood sugar and ketone values were within the normal ranges and propionate feeding caused no appreciable changes.

The average values of the various measurements taken during the course of the experiment are given (Table 1). Milk production and feed consumption were practically identical on the control and propionate feeds. Milk fat percentage and total fat production were reduced by the propionate feeding, but the differences were not statistically significant. Average blood ketone values were slightly lower and there was a very slight weight loss while the cows were being fed the propionate. Again, the small differences were not statistically significant. Most of the cows lost weight during the first 6 wk. of the experiment and gained during the last 6 wk.

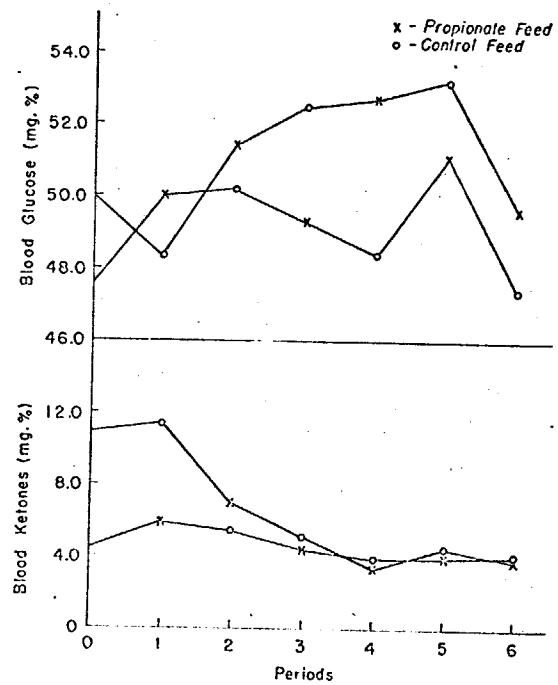

FIG. 2. Effect of feeding ½ lb. of sodium propionate daily during alternate 2-wk. periods on blood sugar and blood ketones.

The results of the supplemental experiment, designed to determine what happened to the fed propionate and how it affected the normal rumen fermentation, appear in Figure 3. The control feed showed the normal curve of fatty acid content in the rumen following feeding, with peak concentration 3 hr. after feeding. When the propionate feed was fed, the level of propionic

acid logically was much higher when the first sample was taken 1 hr. after feeding. It is of interest to note that the levels of acetic and butyric acid were also higher. It is doubtful whether this could be interpreted to mean that the propionate stimulated the production of the other acids, but certainly

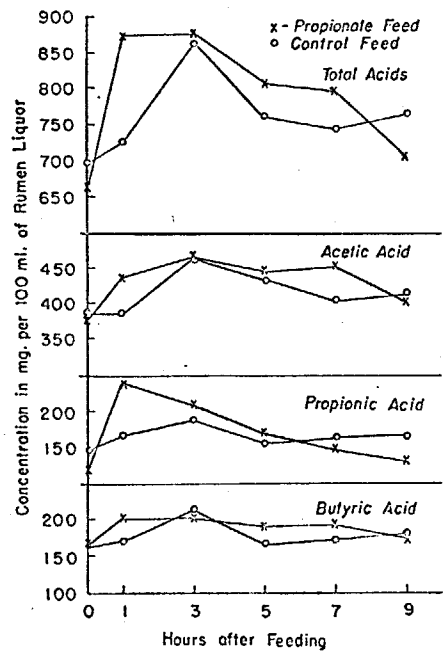

FIG. 3. Effect of feeding 5 lb. of grain containing 5% sodium propionate on volatile fatty acid concentration in the rumen.

it is clear that it did not depress their normal production. After the 3-hr. peak concentration was reached in each case, the curves for the control and propionate feeds followed each other closely. It was calculated that all the administered propionate had disappeared in 7 hr., with most of it disappearing within 3 hr.

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TRANSLATION

ON THE FUNGICIDAL, FUNGISTATIC AND ANTIFERMENTATIVE PROPERTIES OF ORGANIC COMPOUNDS.

Report 1: Sodium Propionate (DuPont Mycoban)

Giorgio Serchi

One of the most urgent needs of the agricultural economy and the foodstuffs economy in particular is that of being able to preserve successfully, for a more or less long period of time, perishable goods, so that they will maintain the original organoleptic properties, without the processes utilized being able to alter the flavor, odor and color of the food or in any way produce any immediate or accumulative toxicity.

Since 1948, with professor R. Poggi and the late professor L. Marimpietri, I have been studying the application, even pharmaceutical, of these preservatives, so that regarding the above-mentioned reason and the individualization of autochthonous and specific preservatives; a long series of experiments, both of scientific and industrial character, has been carried out and is still in the process of execution, experiments on which a report was presented to the XXVIth Congress of Industrial Chemistry this year.(1).

Among the many products studied comparatively by examination, and singly experimented, sodium propionate merits particular mention. this is widely utilized in North America, but up to the present it has not been known and appreciated in Italy. Therefore, it seems useful to me, in this paper, to report on the investigations made, and to contribute to the knowledge of this salt, which, in many ways is preferable to the other derivatives of similar activity already on the market for some time.

It is known that a preservative for food products or for sterilization and therapeutic purposes, must correspond to the following general characteristics:

1. possess polyvalent fungicidal, fungistatic and antifermentative properties, namely, extensive to the majority of those micro organisms(fungi, fungilli, molds or bacteria) to which can be ascribed the alterations of vegetable and also animal foods or the

spoilage of pharmaceutical products;

2. not to present any toxicity to the animal organism, and not to cause, moreover, accumulation phenomena in specific organs or receivers, phenomena which, unfortunately, have been observed for the majority of preservatives used up to the present time, carrying in the molecule aryl residues as well as substitutes (benzoic acid and hydroxy benzoic acids, esters and relative salts, phenols, and phenates, etc.). or highly toxic groupings(nitric group, halogens, etc.);

3. not to alter the organoleptic characteristics of the food with introduction of foreign tastes or odors, even at the concentrations utilized for the success of the preservation. Thus complete inertness is required to even the smallest and frequently volatile components of the food to be preserved or the product to be conserved, besides the conducting and softening liquids which, in every case, must remain clear and unaltered;

4. not to react with the containers or with the wrappings used for containing the foods or products in question, so as to allow also the use in mixings and covering or wrapping suspensions;

5. to have a structure similar to at least one of the components of the food or to the products which it is spontaneously and naturally is capable of forming during the working, cooking and handling processes;

6. to possess a sufficiently prolonged period of action to allow the use of it with confidence even for several years; to be resistant to physical treatments (thermal and irradiation) which can be used for sterilization, after the addition of the preservative;

7. to exert a contrary action not only to the bacterial development but also to the antagonistic substances which many microorganisms are capable of giving off?(secernere - Trans. unavailable), while dying;

8. to be sufficiently economical to allow use on large scale both for low-cost foods and of limited conservability, and for those which a long preservation in time is not rendered necessary, a preservation which could, in some way, compensate for the use of higher cost preservatives.

My work will report on the following fundamental points, trying to classify the activity of the sodium propionate into the general characteristics which I have just summarized above.

A). Chemical and toxicological behavior of sodium propionate compared to other commonly used preservatives.

B). Antifermentative and fungistatic activity of sodium propionate, on cultures in vitro and considered for its possible pharmaceutical application.

C). Sodium propionate as antifermentative product: relative industrial application.

D). Secondary actions of the salt considered on containers normally utilized for the preservation of foods.

A) CHEMICAL AND TOXICOLOGICAL BEHAVIOR OF SODIUM PROPIONATE IN COMPARISON WITH OTHER COMMONLY USED PRESERVATIVES.

Almost all the preservatives used for a long time for the preservation of foods exert a more or less toxic action on the animal organism. Many, even if they do not cause serious disturbances, produce irritations or phenomena of intolerance. Others, and among them also some of very widespread use, accumulate in the organism and are eliminated with such slowness as to risk the normal decrease of the metabolism.

It is for this reason that the health legislation of many countries, and in particular that of Italy, prohibits the use of these products, tolerating them tacitly, however, with the obligation of the declaration, only when their quantity is such as not to be able to be revealed analytically with exactness. This prohibition to the use of preservatives is perhaps too drastic, inasmuch as some really innocucus ones exist, and it leads to the creation of compromises which are not always attractive among the legislators and the industrialists(compromises which force into operation more or less legal machinations which, in final analysis, contribute only to an increase of the fraud. I cite, for example, the use of nitrites and nitrates in the bagging industry and that of copper sulfate in greening liquids for boxed herbage.

Moreover, these difficulties are caused by the necessity of limiting the use of too plainly toxic preservatives, of which, in some nations, too much abuse has indeed been made to the detriment of the consumer. It is, in fact, inconceivable how the toxicity of some compounds used for the preservation of foods is not comprehended which, in many countries which are large consumers of canned foods, are introduced in really sizable quantities.

It suffices to consider the phenols, in general, more or less substituted, however, widely used and for which there are also recent patents (2), salicylic acid and its esters, p-hydroxy benzoic

acid, and derivatives, to repeat with Fabre: "if at the dose employed, phenol derivatives are not capable of causing apparent accidents in the non-sensitized subjects, they are not normal constituents for the organism, and in this respect, they can disturb the metabolism of the living cell" (3).

If then the halogen derivattives are taken into examination (chloroacetic acid, bromacetic acid, chlorophenol and chlorophenolates chloronitrobenzene, dihydroxy-hexachloro-diphenylmethane, etc.), compounds used in many case for the preservation of wines, syrups, and sweet juices in general, and sometimes also (chlorobutanol) for the chemical sterilization of phials, it is obvious from the many scientific publications that they are toxic, that they can be accumulated and that they cause secondary effects which are also very serious. Clarenburg (4), examining, for example, the bromide derivatives, concludes that they, although supplied with a sufficiently high mortal threshold value, can cause, even at rather low concentrations, irrepressible vomiting, and at very low, even minimum dosages serious irritations of the gastric and intestinal mucous membranes.

This is understood without taking account of the subjective sensitizations to foreign derivatives to normal food components, either of the consequent allergic affections, or finally of the relations not yet very clear, but not therefore, negligible, with the "oncogenesi" (translation unavailable).

Therefore, in recent years, experimenters have been particularly interested in the study of some products for which a certain analogy could be foreseen with the food components and with the derivatives of their degradation. Without considering all the research relative to the use of antibiotics, guanyl derivatives (5), dialkyl glycine (6) amino stearic acid (7) etc., all the compounds which are either too sensitive and slightly resistant (antibiotics) or at least so costly as to prohibit the most common applications of them, many derivatives of simple structure, of high preservability and low toxicity have been examined. Among the many which have been considered: propionic acid, succinic acid, adipic acid, undecylene acid, either in the free form or more commonly as salts or esters (8). Already I was concerned in other work on the fungicidal and fungistatic properties of undecylene acids and their salts (9), truly most remarkable properties. It is good, therefore, to observe that said compounds, with more than three carbon atoms, even in small quantities have a flavor and an odor which are not very pleasing, whether used as free acids or whether used as salts, they are not very pure or they tend, through the surrounding or degrading actions, to free a certain quantity of their anionic part.

Considering these premises, more factors have impelled me to a thorough and comparative study of sodium propionate.

1. Its extremely simple structure and the consequent economy of preparation, especially after the recent patents which derive it from propyl alcohol (10).

2. It being a compound easily found in vegetable foods and commonly obtainable during the simplest fermentative processes.

3. The conclusions of the studies of Peck (11) by which a fungicide, of pharmaceutical and clinical application, must not only block the development of the microorganism, but prevent it from producing factors accelerating its own growth. Such research, carried out on trichophyton gipseum and "interdigitale" in Sabourdan broth, with the use of iodine, sodium benzoate, boric acid, sodium undecylenate and crude penicillin in comparison with sodium propionate, alone and in mixture with the corresponding caprylate, have shown how only these last two salts, especially in reciprocal union, and in dose of 5 mg. per 100 cu.cm. of culture broth, can block the development of the bacteria colonies and can impede the formation of trichophyton,factor of intrinsic growth and bacterial production.This property which, in the case in question, places sodium propionate above the other fungicides considered, is presented by crude penicillin and only by it,perhaps in relation to the impurities contained. All the other products have less activity, or as boric acid, can without further ado be classified among the poor preservatives.

4. The lack of a certain organization of the research undertaken up to this time, the absence of Italian studies on the question and especially the necessity of promoting, if necessary, a pharmaceutical application of the product considered.

Detailed information is lacking on the toxicity of sodium propionate and the lack of toxic activity, as well as secondary activities can be inferred, however, from excellent clinical works, which report the posology used. In particular Ocha and Ruiloba (12) report on the therapeutic results of this salt on human mycetoma produced by actynomises mexicanus, particularly satisfactory results; they adopt oral administration of 6 g. for the first week and 12 g. for 15 days more . However, with these truly imposing dosages, secondary actions were not noted.

Therefore, I have felt it would be useful to carry out research on the toxicity of sodium propionate, on the variation of the volatile components in the urine and on the alteration of the quantity of heteroextractable derivatives from some organs, after a considerable prolongation ofadministration.

The tests were carried out on albino rats of average weight of approximately 200 g., kept on standard complete diet, already for 20 days before beginning the experiments. For control purposes, five animals were kept out: those subjected to the experiments, were instead subdivided into three groups, distinguished with the letters A, B, and C.

Group A. - Five animals, subjected to hypodermic administration of 200 mg. per kg. in 20% aqueous solution with doses equal to 0.2 cu.cm. (quantity corresponding to the weight of 200 g.). The treatment lasted for five weeks.

Group B. - Five animals, subjected to hypodermic administration of 200 mg. per kg. in 10% aqueous solution, with doses of 0.4 cu.cm. (quantity corresponding to the weight of 200 g.). The treatment lasted for 5 weeks.

Group C. - Five animals, subjected to initial hypodermic administration, and for 5 weeks, of 100 mg. per kg. in 10% aqueous solution with doses equal to 0.2 cu.cm. Successively for the third, fourth, and fifth and sixth week, the supply was increased weekly to doses of 200 mg., 250 mg., 300 mg. and 350 mg. per kg. in 20% aqueous solution, with doses respectively of 0.2, 0.25, 0.30, 0.35 cu.cm. Said quantities are all related to the average weight of 200g.

Each solution experimented is considered by percentage in volume. With each animal, the urines were collected and examined, as I shall specify, assembling them every five days and comparing the values derived with those obtainable from the analyses carried out on the control animals. On some of them relative to some organs, even the total hetero-extractable derivatives were measured.

During the administration, the normal feeding conditions are maintained, and therefore, identical to the control animals. Daily checks are made of the weight of the animals.

The conclusions of these toxicological experiments, however incomplete and still requiring amplification, can be summarised in these fundamental points:

a. no animal, among the various groups treated, for the doses experimented, attained the mortal threshold.

b. no noticeable variations in the weight of the animals treated were found with respect to the controls. The positive and negative differences, rather small, can perhaps be attributed to the normal phases of the bodily development, to the feeding and to assimilation. Graph.no.1 shows the ponderal data for some of the animals in question, compared to those obtained with the controls.

c. no reactions of cutaneous sensitization were noted. However, after four weeks of treatment, for the animals of group B, perhaps in relation to the quantities of liquid injected, a slight local falling of the hair is noted. This falling stops, and the pelt takes on its normal appearance again, 15 days after the end of the treatment.

d. in the rats of group B, in the fifth and sixth week, the formation of small subcutaneous abscessual protuberances was noted, in relation perhaps with the large quantity of salt accumulated locally, protuberances which are rapidly reabsorbed.

e. The analysis of the urines was conducted relative to the volatile components, subjecting them to a very slow distillation, on water-bath and collecting the distilled products in a diuretic containing a considerable quantity of NaOH N. 100 . Each urine collected is neutralized with dilute sulfuric acid, before subjecting it to distillation, for the purpose of settling the volatile bases which could be carried along by the distillate, and consequently, falsify the measurements of uncombined soda.The results, obtained every five days for all the controls and for some of the rats under toxicological examination, are given in 10 cu.cm. and reported in acetic acid in mg. The graph no. 2 shows some eliminations curves.

From the data given, it is noted how, while in the controls the content of volatile products of acid character undergo positive and negative variations of 3% at the maximum, in the treated rats, respectively of the group A, B and C, the same content is increased respectively by 11%, 16.5%, and 9%. This increase is manifested generally after the tenth day with rapid ascent of the relative quantities; it stabilizes regularly by the third to the fourth week, and tends finally, in some cases, also to decrease. This report seems to indicate that a part of the sodium propionate is eliminated through the urine, in the form of volatile components, without undergoing any preventive blocking in the organism. This does not exclude that eliminations are also checked in the form of derivatives no more acid, such as salts or esters.

f. On some rats, for each group, the content of hetero-extractable products of some organs (liver and adrenal glands) was determined, always at the end of the treatment and in reference to the controls. A part of the organ under examination is disintegrated in water with quartz sand, filtered quantitatively by pump under slight suction on a large size glass diaphragm, to eliminate the water soluble components and then extracted in soxhlet for 24 hours with absolute ethyl ether. The ethereal solution, preventively dried on sodium sulfate, is treated analytically in analogy with the research of fatty acids.

A second quantity also disintegrated in quartz sand and also filtered is preventively treated when hot, for about two hours with NaOH 2 N, then recovered with water and acidified by a second filtration. The residue is also extracted with anhydrous ethyl ether. The solution acidified in congo red with sulfuric acid, is shaken several times in separating funnel with absolute ethyl ether, until obtaining perfectly clear and colorless upper levels. The two ether solutions, whether from the residue or from the solution, are treated similarly to the preceding one.

I realize that these measurements err in inexactness both quantitative and constitutive, but they can supply values of a certain interest, in so far as carried out in relation to the control, always with the same procedure.

By examination of the above reported data, it is evident how, by acting on rats of the same bodily weight, and thus with almost identical weight of the organs examined, considerable lacks of equilibrium are noted in the content of soluble ethereal substances either in the liver or in the adrenal glands. All this without any lack of equilibrium of the feeding but with simple administrations of sodium propionate. I note this fact without giving an interpretation to the phenomenon, until it can be made the result of a much larger number of experimental observations. In any case, the variations found are all constantly positive and attain considerable differences with oscillations from a minimum of about 30% to a maximum of 90% in regard to the saponifiable derivatives of the liver and from a minimum of 20% to a maximum of 40% for the non-saponifiable derivatives of the liver. For the adrenal glands, the variations are somewhat more limited, but equally noticeable.

B) FUNGICIDAL ACTIVITY OF SODIUM PROPIONATE IN VITRO.

The studies on the fundicidal activity of sodium proprionate are not numerous and are discontinuous, and in many cases carried out on microorganisms determining the most common feeding alterations. Thus, the studies of Keeney (13) relative to the inhibition of bacterial development on the part of the salt used, show that one of his solutions of 1.25% is fungistatic for trichophyton purpureum, for epidermophyton inguinale, for Microsporum Audouini, for Candida albicans, for torula istolitica, for coccidiodes immitis, for aspergilli fungicatus and flavus: a 0.125% solution is fungicidal for trichophyton gipseum, vioaceum, epidermophyton rubrum and interdigitale for microsporum lanosum and for trichophyton schoenleini, whereas a solution at 0.0125 is active on blastomices dermatitidis at 2% sodium propionate inhibits actinomices bovis. On inhibition to the development of Escherichia coli and on protective action exerted on it by beta alanina , the researches of Wright and Briggs (14), as well as Clapper (15), are worthwhile. With regard to the

action exerted on the respiration of fungi by sodium propionate, I recall the research of Nickerson (16) who noted on Epidermophyton fioccosum, treated with 0.01 molar solution of the salt in plug of KH_2PO_4, a depression of the impelled respiration up to 35%.

These studies give importance to the fungicidal activity of the product examined on a rather large number of microorganisms. It appears very wide so that the lack of pharmaceutical applications in Italy is surprising, applications which in other countries become more and more extended up to patenting protective adhesive bands containing from 3 to 10% of sodium propionate (17), and the affirmation by Heseltine and Galloway that sodium propionate is one of the most potent inhibitors of bacteria and fungi responsible for local and more serious infections.

Therefore, I carried out a determination of the bactericidal and bacteriostatic power of aqueous solutions of this salt. Each experiment was conducted on thin plate and precisely on the following types of microorganisms:

- Staffilococcus albus and aureus:
cultivated in broth of agarized meat at 1.5% ;

- Pseudononas aeruginosa: Salmonella thyphi, parathyphi A and B ;

Bacterium antracis: Shigella shigae: Mycrobacterium tuberculosis (stem H_37):
also raised on infusion and broth of agarized meat:

- Saccaormyces cerevisiae var. ellipsoideus (Hansen) and var. turbidan (Hansen):
raised on Hansen liquid of peptonized maltose;

- Lactobacillus casei (Orla Jensen):
raised on peptone agar:

- Thermobacterium bulgaricum (Orla Jensen):
isolated on Markoff earth: (yeast waterwith meat extract, yoast autolysate, disodium phosphate, sodium chloride and agar in the quantity of 2.5%).

This selection was occasioned by the necessity both of giving a rather broad picture of the activity of sodium propionate and of extending this research also to non pathogenic microorganisms, which are capable of determining food fermentation.

Aqueous solutions of the following concentrations were utilized: 2%: 1%: 0.5%: 0.2%: 0.1%: 0.05%: 0.02% .

The addition to the culture is made on support of phosphatic agar continuously utilized for each experiment, adding, by volume, 2% of the solutions under examination to the substrate already prepared. On it are sown the cultures preventively selected and enriched and the entire thing is placed in thermostat at 35°, leaving it in observation for ten days, and noting the behavior of the bacterial development every 24 hours.

I have considered as fungistatic those concentrations which at least for 48 hours impede the growth; those which inhibit the development throughout the entire period of observation are fungicides. The results can be thus indicated:

- The 2% solution is bacteriostatic for pseudomona aeruginosa and shigella shigae (prevention for 72 hours): it is a bactericidal for saccaromyces and lactobacillus. It impedes the development of all other microorganisms considered for 24 hours.

- The 1% solution preserves its activity on saccaromyces and on lactobacillus: for 24 hours it blocks the development of all the other microorganisms exclusive of Mycobacterium tuberculosis and staffilococchi.

- The 0.5% solution is bacteriostatic, with prevention up to 120 hours, with reference to saccaromyces and lactobacillus: it impedes the development of salmonelle and Shigella for 24 hours. It exerts no activity on the other microorganisms.

- The 0.1 and 0.05 solutions block the development of saccaromyces and lactobacillus for 72 and 24 hours respectively. They are not active on the others.

- The 0.02% solution is completely inactive.

The observation of a good fungistatic and also fungicidal activity has been noted on microorganisms of lactic and alcoholic fermentation, which impelled me to do a series of research on the preservation of fermentable sugar liquids, by means of aqueous solutions of sodium propionate. Such research, concluded initially in the laboratory, has been extended also to the industrial plane.

C. ANTIFERMENTATIVE ACTIVITY OF SODIUM PROPIONATE ON FERMENTABLE SUGARED LIQUIDS.

The use of fatty acids (propionic, butyric, isobutyric, glycolic, lactic, hydroxy-isobutyric, α- and β-hydroxybutyric) and their salts

as retarders of alcoholic fermentation, was proposed until 1927 by Katagiri (19), but was taken up again with success, however, in the last ten years, and has affected the conservation of many foods, and the impedence of the fermentative actions which can easily alter them.

Frank and Hess (20) examined and advised the use of sodium propionate for the control of the development of the growth of sporendonema epizoum in sodium chloride for salting fish.

Wolford and Andersen (21) report optimum methods for conservation of fruit (figs, blackberries, pomegranates, etc.) or vegetables (unshelled peas and beans) by spraying and immersion in solutions of sodium propionate of concentrations varying between 5 and 10%. Roberts (22) examined the action of this salt in the preservation of bread and the percentual variations which are derived in comparison to the baking and the drying. Schelhorn (23) considers sodium propionate as the best inhibitor of saccaromices, fungilli, escherichia, and pichae, also in comparison to the other products of similar action: salicylic acid, p-hydroxy-benzoic acid, p-chloro-benzoic acid, formic acid: ethyl esters of p-hydroxy-benzoic acid. Very recently then Cornwell (24) patented the use of an aqueous solution of hydroxyethyl or methyl cellulose, ethyl cellulose, its esters with glycolic acid, bearing freed sodium propionate as covering and preserving means for meat, fruit, and vegetables in general. Similarly, Darrell & Downs (25) recommend the use of said salt in the manufacture of flexible and elastic covering for cheeses.

My experiments on the question were carried out with the following food products:

- Apple juice D 1,112 containing 13.7 of sugars:
- Sweet filtrate of grape D. 1,106 containing 16.3% of sugars:
- Orange juice separated from the pulp by filtration D 1,097 at 7.2% of sugars.

The juices in question were preserved at temperatures between 20 and 30 degrees, that is, at the optimum temperature for fermentation, in clear glass bottles, without any preventive sterilisation and without hermetic closing. In each container examined (10 for each juice experimented) was added, to groups of two, 0.2:0.4: 0.6: 0.8; 1% by weight of sodium propionate, in 25% aqueous solutions.

The substances under examination were kept for about 10 months and controlled either organoleptically or with respect to their sugar content.

The concentrations equal to 0,2: 0.4: and 0.6% were not active or however, not applicable industrially. In fact, the control carried out, either by the calculation of the carboxylic anhydride fixed on standard containers containing NaOH, or by the decrease of the percentage of sugar present in the liquid under examination, has shown that the above mentioned concentrations block fermentation respectively for 5,11,and 21 days, keep it very slight for a week, but later exert almost no action.

Identical results are obtained with solutions at 0.8 to 1%. Fermentation is completely blocked by about 90-110 days. The development of carboxyclic anhydride in said period is minimum or absolutely nil. The quantity of sugar fermented after 30,60, 90,120, 150 days is decreased according to these values:

CONTROLS:

Apple juice: Fermentation begun after 3 days: maximum development of CO_2 from sixth to 20th day; decrease glucides: 65%; 71%; 74%; 76%; 77% ;

Sweet filtrate: Fermentation after 24 hours; almost immediate development of CO_2; maximum from first to seventh day; decrease glucides: 83%; 85%; 88%; 89%;

Orange juice: Fermentation after 36 hours: maximum development of CO_2 from second to sixth day; decrease glucides: 47%; 49%; 51%; 52%; 54%.

TREATED WITH 1% OF 25% SOLUTION OF PROPIONATE:

Apple juice: Fermentation begins on the 94th day, proceeds slowly for 14 days with minimum development of CO_2; becomes violent from 110th to 117th day. The sugar undergoes these percentages of decreases: 3.7%; 4.6%; 4.8%; 47%; 63%.

Sweet filtrate: Fermentation begins only on the 115th day: it is very slow for another 14 days; then it proceeds rapidly from the 115th to the 125th day. The percentage of glucides undergoes the following variations: 1.1%; 2.05%; 3.32%; 52%; 78%;

Orange juice: Fermentation begins only on the 130th day; it is immediately violent and ends after about 10 days; the percentage of glucides diminishes by 0.7%; 1.1%; 1.8%, 2,7%,56%, 58%.

There are not notable organoleptic variations in the juices preserved. Only the orange juice has a slightly rancid flavor, perhaps due to the oxidizing action of the essentialoils contained in the peel and which can be added to the juice during the squeezing.

Such a difficulty could be avoided by the addition of one of the many antioxidants commonly used in commerce.

The tests of industrial character, to which I shall refer in due course, have concerned: blocking of fermentation of sweet filtrates and of pressed grapes in substitution of the SO_2 or of the metabisulfites; activity on the fermentation of bread for a long period of time, also in relation to the baking and to the flour used: experiments on the preservation of fruit juices and pulps.

However, reserving my right to extend them and to characterize them on other products, this antifermentative activity has given very favorable results sufficient to recommend a true and sizable application of sodium propionate in even sizable doses.

The same may be said for some pharmaceutical applications either in the form of an ointment for use on the skin; or as sterilizing solutions for surgery.

SUMMARY

After having given a picture of the studies which have preceded the research on the matter in question, studies which bring out the importance of it in the fields of nourishment and pharmacy, the author examines from various viewpoints the use of sodium propionate.

The absolute harmlessness of it is established even in really imposing doses, especially in comparison with the other preservatives commonly used. One of its eliminations through the urine is observed with discovery of the increase of volatile components; a positive variation of the hetero-extractable saponifiable and non-saponifiable components is observed in the liver and in the adrenal glands, after administration of sodium propionate. The fungistatic and fungicidal activity of it is documented on a good number of pathogenic or fermenting microorganisms, by means of experiments carried out on plates. The preservations of fruit juices (orange, grape and apple) is studied with the use of 0.8 and 1% of aqueous solutions of sodium propionate. Proposal is made to extend the industrial application of it either as food preservative or as product of pharmaceutical importance.

Table 3

Content in hetero-extractable substances without or with saponification of the liver and adrenal glands of some animals under examination.

Rats	LIVER			ADRENAL GLANDS		
	without saponification	with saponification solution	with saponification residue	without saponification	with saponification solution	with saponification residue
A_1	123	720	114	26	53	31
A_2	115	812	131	17	64	22
B_3	78	652	94	21	76	15
C_2	110	947	120	11	83	16
1	83	503	71	15	51	10
2	92	526	106	16	48	10

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CAGLIARI - Istituto di Chimica Farmaceutica dell'Università -
November 15, 1953.

Tra[illegible]y

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DECOMPOSITION

Separation and Identification of Sodium Salts of Acetic, Propionic, Butyric, and Valeric Acids by Paper Chromatography

By JAMES A. YOUNG, GEORGE SCHWARTZMAN, and ANNA L. MELTON (Food and Drug Administration, 1141 Central Parkway, Cincinnati, Ohio, 45202)

A method has been developed to confirm the identity of volatile fatty acids after they have been steam distilled, separated by a silicic acid column, and titrated with NaOH. The method works equally well for the detection of propionates in bread products. This paper reports a second collaborative study with a modified mobile solvent: two volumes of acetone added to equal volumes of *n*-butanol, tertiary butanol, and concentrated NH_3. This solvent system gives a more distinct separation of the acid spots, the spots remain stable longer, and the time required for the paper to develop is reduced to 2.5 hours. Six laboratories and eight collaborators participated in the study. The method was adopted as official, first action for identifying the sodium salts of the C_2–C_5 acids and as a qualitative test for propionates in bread products.

In a previous collaborative study on the identification of volatile acids (1, 2), two of the five collaborators failed to identify all the unknowns. To remedy the situation, additional work was done in this laboratory to improve the solvent system.

Excellent results were obtained by adding two volumes of acetone to the solvent system used in the original method (1). This addition produced a chromatogram, in about 2.5 hours, with distinctly separated spots that remained stable longer.

Use of the modified solvent system permitted identification of the C_2–C_5 acids after they had been steam distilled, separated on a silicic acid column, and titrated with NaOH (3). The method is also an excellent technique for the detection of propionates in bakery products. The new solvent system showed that sodium benzoate or sodium sorbate will not interfere with the detection of propionates; the R_f values of these preservatives are higher than those of propionates. Thus, propionates can be detected in the presence of the sodium salts of the other two preservatives.

Six laboratories and eight collaborators participated in this second collaborative study. Six samples were prepared to contain various mixtures of the sodium salts of the C_2–C_5 volatile fatty acids. Two bread samples were also prepared; one contained 0.1% sodium propionate. In addition, two "known" vials of the salts were forwarded so collaborators could become familiar with the method. The collaborators were asked to analyze these "knowns" before checking the unknowns and the bread products.

The following method was sent to the collaborators:

METHOD

Apparatus

Chromatographic tank and accessories.—See 24.098.

Reagents

(a) *Mobile solvent.*—Mix 2 vols acetone, 1 vol. *tert.*-butanol, 1 vol. *n*-butanol, and 1 vol. NH_4OH. Prep. fresh daily.

(b) *Chromogenic reagent.*—Add 200 mg each Me red and bromothymol blue to mixt. of 100 ml formalin and 400 ml alcohol. Adjust to pH 5.2 with 0.1*N* NaOH.

(c) *Standards.*—Pipet 1 ml each acetic, propionic, butyric, and valeric acids into sep. 100 ml vol. flasks and dil. to vol. with H_2O to prep. stock solns. Pipet 1 ml each of these stock solns into sep. 25 ml beakers; pipet 1 ml each of the 4 stock solns into single 25 ml beaker to prep. known mixt. Neutralize acids and mixt. with 0.1*N* NaOH, using cresol red indicator, 15.127(f), avoiding excess alkali. Evap to ca 0.5 ml, or evap. just to dryness and take up in 0.5 ml H_2O.

This report of the Associate Referee, James A. Young, was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct 19–22, 1964, at Washington, D.C.

Determination

Steam distill
13.083, collectin
ately neutralize
15.127(f), and
or evap. just to
0.5 ml H_2O. (T
large porcelain
ml; transfer to
0.5 ml.)

With hard per
bottom edge of
washed chromat
µl spots with 1
leaving 1" margi
acid, one spot f
unknowns. Spot
propionic, unkn
butyric, valeric.
rod and suspend
solvent in troug
heavy liquid, us
glass rod to prev
uniform solvent f
mobile solvent b
glass cover with
tape and let dev
from top of pape
let air-dry, and s
mogenic reagent.
and rather heavy
mogenic reagent
spots indicate pre
spots are due to
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mentarily (e. g., b
in 2 L beaker, ro
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ing their R_f valu
individual acids.

Since color of ac
with pencil as soon
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Summary an

All collaborator
rectly identified th
ous volatile acids i
also the bread sam
propionate (Tabl
stated that the m
follow, and accura

Determination

Steam distill 20 g well-mixed sample as in 13.083, collecting 200 ml distillate. Immediately neutralize distillate, using cresol red, 15.127(f), and 0.1N NaOH. Evap. to 0.5 ml, or evap. just to dryness and then take up in 0.5 ml H_2O. (To save time, place distillate in large porcelain evapg dish, and evap. to 5–10 ml; transfer to 25 ml beaker and evap. to 0.5 ml.)

With hard pencil, rule starting line 1″ from bottom edge of 8 × 8″ Whatman No. 1 unwashed chromatographic paper. Spot seven 1 µl spots with 1 µl pipet on paper, 1″ apart, leaving 1″ margin, to permit 1 spot for each acid, one spot for mixt., and two spots for unknowns. Spot in following order: acetic, propionic, unknown, mixt. of 4, unknown, butyric, valeric. Let dry. Clip paper to glass rod and suspend in tank with 50 ml mobile solvent in trough. (Since mobile solvent is heavy liquid, use 3 clips to fasten paper to glass rod to prevent sagging and to give more uniform solvent front. Do not sat. tank with mobile solvent before inserting paper.) Seal glass cover with cellophane or other suitable tape and let develop until solvent is ca 1″ from top of paper. Remove paper from tank, let air-dry, and spray on front side with chromogenic reagent. (Spraying should be uniform and rather heavy but not to extent that chromogenic reagent runs or drips.) Faint yellow spots indicate presence of acids; heavier blue spots are due to Na ion. To intensify acid spots, place paper in atm. of NH_3 fumes momentarily (e. g., by placing ca 50 ml NH_4OH in 2 L beaker, rolling paper, and exposing to fumes by placing each end in beaker momentarily); entire paper immediately turns green. Remove paper from NH_3 fumes. Acids gradually appear as red spots, and presence of specific acids in sample may be detd by comparing their R_f values with those of mixt. and individual acids.

Since color of acids is not stable, mark spots with pencil as soon as they are completely developed.

Summary and Recommendations

All collaborators in 6 laboratories correctly identified the sodium salts of the various volatile acids in each vial (Table 1), and also the bread sample containing the sodium propionate (Table 2). The collaborators stated that the method was clear, easy to follow, and accurate.

Based on the results of this study, it is recommended that this method be adopted as official, first action for confirming the identity of volatile fatty acids (C_2–C_5) after steam distillation, separation by column partition chromatography, and titration with NaOH (3); and for the qualitative detection of propionates in bread products.

The Associate Referee plans further studies of this method to develop a screening technique for the detection of decomposition in food products. The possibility of using this method to detect propionates in such

Table 1. Collaborative results for determination of C_2–C_5 fatty acids; 8 collaborators

Vial No.	Contained Sodium Salts of	Collaborators Recovering Fatty Acids Present
1	Propionic Valeric	All 8
2	Butyric Propionic Valeric	All 8
3	Acetic Propionic Butyric Valeric	All 8
4	Butyric Propionic	All 8
5	Acetic Propionic	All 8
6	Acetic Butyric	All 8

Table 2. Collaborative results for determination of C_2–C_5 fatty acids; 6 collaborators reporting

Bread Sample No.	Contents	Propionates Recovered
1	No propionates	None
2	Propionates	All

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee C, and were adopted by the Association. See *This Journal*, 48, 172 (1965).

products as fruit pies, cream filled pies, etc., will be explored.

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Ammonia as an Index of Decomposition in Crabmeat

By JAMES L. BURNETT (Food and Drug Administration, 501 Federal Office Building, Seattle, Wash. 98104)

A method, based upon the color reaction between NH_3, thymol, and bromine, uses ammonia as an index of decomposition in fresh or frozen crabmeat. The color is extracted into *n*-butyl alcohol and net absorbance is determined by subtracting absorbance at 475 mμ from that at 682 mμ. Sensitivity is about 1 ppm NH_3. The method is very rapid and requires no special apparatus. Samples of crabmeat decomposed under controlled conditions were examined and graded organoleptically. Ammonia content was determined and found to increase uniformly and rapidly with spoilage. Good reproducibility and low results were obtained on fresh crabmeat. Ammonia can be detected by the method before spoilage can be detected organoleptically.

Various methods for determining ammonia have been proposed to detect decomposition in fishery products. None of these has been successful as accurate quantitative methods, although a recent qualitative test using a modified Nessler's reagent has been devised (1).

Since ammonia is formed quite rapidly and in fairly large amounts during shellfish decomposition, a study was undertaken to find a reliable quantitative method for ammonia in crabmeat.

Nessler's reagent can be used as a qualitative test, but a precipitate forms which makes the test unsuitable for quantitative measurement. To overcome this, a modified method, involving a color reaction with thymol and bromine water (2), was proposed to measure ammonia.

Fresh boiled Dungeness crabs were placed in the laboratory at room temperature (24°C) and allowed to spoil. Seven whole crabs were cracked daily, and the meats were ground together and frozen after organoleptic judging. The crabs were judged class 0 (fresh), class 1 ("fishy," still good), class 2 (slight but definite decomposition), class 3 (advanced decomposition), or class 4 (putrid). These organoleptic classes conform to previous definitions (3).

The proposed method is very rapid (about 30 minutes to complete a series of determinations) and requires no distillation or special apparatus. Sensitivity is about the same as with Nessler's reagent (about 1 ppm NH_3 in crabmeat).

METHOD

(Use NH_3-free water thro distilled water is suitable.)

Reagents

(a) *Bromine solution.*—Dis mine in 100 ml water. Add and mix. (Solution is stable stored in dark bottle.)

(b) *Thymol solution.*—Diss in 10 ml 2N NaOH, and dilu water. (Solution is stable f stored in dark bottle.)

(c) *Ammonia standard sol* 0.314 g NH_4Cl, previously dri in water and dilute to 100 r ml to 200 ml volumetric flas volume with water. 1 ml = 5

(d) *Phosphotungstic acid.*— lution (w/v).

Preparation of Samples

Remove meat from shell (grind 3 times through food after each grinding.

Determination

Place 50 g prepared sample 100 ml 10% phosphotungstic ac and blend about 2 min. at lo through fluted filter paper (S equivalent) into 250 ml E collecting first 20 ml in separa returning to filter paper (first cloudy).

Pipet 25.0 ml filtrate (rep crabmeat of 78% moisture) int meyer flask, and neutralize to adding 10% NaOH dropwise excess. Add 10.0 ml thymol so mix. (Ignore any white precipi form.) Add 5.0 ml bromine sol and let stand 20 min. (Blue so decomposition.)

Transfer solution to 125 ml Rinse flask with 2–5 ml ding rinsings to funnel. Add ohol (reagent grade), and sh sec. Discard lower aqueous l ol layer through 1" column meter) of anhydrous Na_2S pered flask.

Pipet 5.0 ml into suitable equal volume of *n*-butyl alc mine absorbance at 475 a cell, using *n*-butyl alcoh Final color solution is stabl

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REPORT OF A STUDY ON THE TOXICITY OF SEVERAL FOOD PRESERVING AGENTS[1]

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The deterioration of some foods caused by the activity of microorganisms may be prevented and the keeping quality of the foods greatly improved by the addition of certain chemical compounds. It is obvious that preservatives which produce a marked toxic effect should not be used in food intended for human consumption. On the other hand, even when a marked toxicity is not shown, the consumption of food containing unusual quantities of preservatives may be dangerous and harmful because of the hidden cumulative effect.

Definite proof of toxicity lies in knowing how the chemical agent is handled in the body. By tracing the chemical reactions of the preserving agent in the animal body and determining the method by which it is eliminated from the body, an accurate statement concerning its toxicity can be made.

This experiment was designed to yield information that may contribute to the knowledge of the toxic properties of certain chemical substances used in human food, and a bactericidal material used for sterilizing utensils. The amount of gain made by growing white rats was used as an indication of the toxic effects. Individual feeding trials were conducted with diets to which were added calcium propionate, sodium propionate, sodium benzoate, zephiran, and sodium benzoate plus glycine. Propionates are used in the bread industry and to some extent in packaged cheese in order to control mold. Zephiran is a new germicide which may be used for disinfecting dairy utensils. Sodium benzoate has been used for some years as a preservative for various food products.

Griffith (1) has shown that when 1.5 per cent or more sodium benzoate was in the diet, the rate of growth in young rats was decreased. The excretion of hippuric acid in the urine accounted for 66 to 95 per cent of the benzoic acid consumed as sodium benzoate. Small doses of sodium benzoate gave nearly perfect quantitative recovery of hippuric acid. As the amount of sodium benzoate was increased, the percentage recovered as hippuric acid decreased. It was assumed that this showed a lack of sufficient glycine in the animal for the detoxication of the benzoic acid. The addition of glycine

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[1] This study was a graduate research problem conducted under the direction and supervision of Professor T. S. Hamilton, Department of Animal Husbandry, University of Illinois.

to the diet containing sodium benzoate increased the growth rate and decreased the toxic effects.

Rittenberg and Schoenheimer (4) showed that animals use tissue glycine in preference to dietary glycine for the detoxication of benzoic acid. About two-thirds of the nitrogen in the hippuric acid excreted was not of dietary origin. An excess of dietary glycine did not change this ratio.

PLAN OF EXPERIMENT

White rats were fed diets containing calcium propionate, sodium propionate, sodium benzoate, zephiran, and sodium benzoate plus glycine. Feeding of the experimental diets started when the rats were about four weeks of age and continued for four to five weeks. The paired-feeding technique (2, 3) was used, with a modification so that one control animal was compared with two experimental animals. Eight triplicates (24 rats) were fed during each part of the experiment. In each triplicate, litter mates of the same sex were used and the maximum weight variation of the young rats at the start was 4 grams.

In series I the control animals were fed a basal diet containing calcium lactate, a second group was fed diets containing 1 per cent and 3 per cent calcium propionate, and a third group was fed diets containing 1 per cent and 3 per cent sodium propionate.

In series II the control diet contained calcium lactate, one experimental diet contained 3 per cent sodium benzoate, and the other experimental diet contained 3 per cent zephiran, a germicidal agent. Zephiran is sold as a ten per cent aqueous solution of a mixture of high molecular alkyldimethylbenzylammonium chlorides. When used as a rinse for utensils, 1 part of zephiran is added to 5,000 to 10,000 parts of water.

In series III the control diet previously used was compared with an experimental diet containing 1 per cent sodium benzoate and another containing 3 per cent sodium benzoate plus glycine. Theoretically, .52 gram of glycine is required to detoxify 1 gram of sodium benzoate, but one gram of glycine was fed for each gram of sodium benzoate in order to supply more glycine than was needed.

In series IV all the rats used in series III were fed a diet containing 3 per cent sodium benzoate. The same grouping was maintained in order to study the residual effect of feeding small quantities of sodium benzoate and also of feeding glycine.

The composition of the diets used in each series is given in table 1. Waste by rats was prevented by mixing enough distilled water with the feed at feeding time to give the mixture the consistency of paste. Distilled water was supplied at all times in individual water fountains.

RESULTS

Comparisons of control rats with experimental rats showed that calcium

propionate, sodium propionate, and zephiran fed at the levels used in these trials did not decrease the amount of gain. Sodium benzoate fed at the rate of 3 per cent proved harmful: the amount of gain was much less than for the control group, and two rats out of eight died before the end of the trial.

TABLE 1

Composition of diets

Series and group	Constituents of each diet					
	Fox chow	Yeast	Cod-liver oil	Salt	Calcium lactate	Experimental substance
	%	%	%	%	%	%
Series Ia:						
Control	91.5	5.0	2.0	0.5	1.0	....
Ca propionate	91.5	5.0	2.0	0.5	....	1.0
Na propionate	91.5	5.0	2.0	0.5	....	1.0
Series Ib:*						
Control	89.5	5.0	2.0	0.5	3.0	....
Ca propionate	89.5	5.0	2.0	0.5	....	3.0
Na propionate	89.5	5.0	2.0	0.5	....	3.0
Series II:						
Control	89.5	5.0	2.0	0.5	3.0	....
Na benzoate	89.5	5.0	2.0	0.5	....	3.0
Zephiran	89.5	5.0	2.0	0.5	....	3.0
Series III:						
Control	89.5	5.0	2.0	0.5	3.0	....
Na benzoate	91.5	5.0	2.0	0.5	....	1.0
Na benzoate plus glycine	89.5	5.0	2.0	0.5	....	3.0‡
Series IV:†						
Na benzoate	89.5	5.0	2.0	0.5	....	3.0

* Rats continued from series Ia.
† Rats continued from series III.
‡ One part of glycine was added for each part of sodium benzoate.

For the group of rats receiving 3 per cent sodium benzoate, the average amount of gain produced per 10 grams of feed consumed was 1.01 grams smaller than the average for the control group. This is considered a significant margin. The addition of glycine to the 3 per cent sodium benzoate diet reduced the toxic effect of the benzoic acid. The difference between the amount of gain produced per 10 grams of feed consumed when glycine was fed was only .24 gram in favor of the control group. This fact is probably significant, however, because the gain of the control individual over that of the rat receiving sodium benzoate plus glycine was consistent for all pairs tested. This result may indicate a limited capacity of the animal organism to synthesize glycine or to utilize dietary glycine for the detoxication of sodium benzoate.

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TABLE 2

Grams of gain produced

Series and groups	Gain by triplicates 1	2	3	4	5	6	7	8	Total gain	Average	Difference	S.D.	P. value	Odds	Gain/10 gms. feed
Series Ia (4 wks.)															
Control group	88	82	70	86	88	60	78	80	632	79.0					3.34
Ca propionate	90	82	66	87	91	62	82	80	646	80.0	+ 1.0	2.29	.1429	1:7	3.38
Na propionate	85	84	67	86	87	67	80	82	638	79.8	+ .75	2.11	.1881	1:5	3.37
Series Ib (3 wks.)															
Control group	50	26	30	50	43	20	33	36	288	36.0					1.74
Ca propionate	52	16	34	50	39	17	36	35	279	34.9	− 1.12	4.25	.2586	1:4	1.68
Na propionate	53	17	35	51	36	25	43	33	293	36.6	+ .63	6.08	.3994	1:3	1.77
Series II (4 wks.)															
Control group	63	70	65	74	69	65	74	77	577	69.6					3.24
Na benzoate (3%)	51	53	58	*	*	59	32	32	285	47.5	−21.5	15.98	.0148	1:68	2.23
Zephiran (3%)	*	71	70	76	80	62	77	72	509	72.7	+ 2.1	4.67	.1525	1:7	3.35
Series III (5 wks.)															
Control group	82	100	98	103	97	100	98	98	776	97.0					3.12
Na benzoate (1%)	87	96	98	97	95	97	97	102	769	96.1	− .88	3.55	.2666	1:4	3.09
Na benzoate (3%) plus glycine	74	87	90	94	95	92	90	95	717	89.6	− 7.38	3.23	.0002	1:5000	2.88
Series IV‡ (2 wks.)															
Group 1	18	22	*	23	19	26	28	26	162	23.1					1.33
Group 2	32	20	28	27	23	33	22	29	214	26.8	+ 3.43	5.90	.1033	1:10	1.55
Group 3	32	24	29	*	25	32	30	22	194	27.7	+ 4.33	5.47	.0668	1:15	1.61

* Omitted because of incomplete results.
† Mean difference between control group and experimental group.
‡ Rats of group 1 continued from control group of series III.
Rats of group 2 continued from Na benzoate (1%) group of series III.
Rats of group 3 continued from Na benzoate (3%) plus glycine group of series III.

At the termination of the tests in series III, all the rats of that trial were changed to a diet containing 3 per cent sodium benzoate. Results obtained from this diet were compared with those of previous diets fed, but the probable error was large. The control group which had never received any sodium benzoate was most seriously affected by the change in diet. This group consumed 7.49 grams of feed per gram of gain compared to 6.44 for the group which had previously received 1 per cent sodium benzoate and 6.20 for the group which had previously received 3 per cent sodium benzoate plus glycine.

These results indicate that the group of rats which had previously received glycine had more resistance to the toxic effect of sodium benzoate than any other group. The utilization of stored glycine may explain the increased resistance. However, it seems reasonable to assume that the glycine reserve of group 2, which had received previously sodium benzoate and no glycine, should have been depleted and that additional quantities of sodium benzoate should have been very toxic. Instead, this group of rats made a greater gain than group 1 and practically as much gain as group 3. This fact may indicate that with limited feeding of sodium benzoate a mechanism was set up which enabled the animals to more readily combat toxicity.

Series II gives further evidence that rats which consume sodium benzoate develop a resistance to its harmful effects. The symptoms of toxicity were evident and the deaths caused by it occurred 7 to 20 days after the start of the trial. The rats which lived longer than 20 days showed an increasing resistance to the toxic effects of sodium benzoate.

The toxicity of sodium benzoate was expressed by definite, characteristic symptoms. The animal first became irritable and rigid, responding to the slightest touch or disturbance by biting the cage or the handler. As the symptoms progressed, it lost its power of coordinated movements and had convulsions.

A summary of the grams of gain produced in each part of this experiment is presented in table 2.

SUMMARY

A study was made of the amount of gain produced in growing white rats which were fed diets containing chemical agents used in food products or as a sterilizing agent. The amount of gain produced was used as an indication of the toxicity of these chemical agents. It is recognized that the method of measuring toxicity by the amount of gain produced has a limited application.

Neither calcium propionate nor sodium propionate, when fed in quantities up to 3.0 per cent of the diet, decreased the amount of gain.

Zephiran, a disinfectant made up of high molecular alkyldimethylbenzylammonium chlorides, when included in the diet at the rate of 3.0 per cent,

did not cause any ill effect. In fact, the zephiran group made slightly greater gain than the control group; and out of seven pairs of rats finishing the trial, five of the rats receiving zephiran made greater gains than their paired members in the control group. The results of this study indicated that zephiran is not toxic, and since it is a germicide there is a possibility that it could be employed to help control the bacterial flora of the alimentary canal in experiments where that feature is desired.

Sodium benzoate fed at the rate of 1.0 per cent in the diet did not affect the amount of gain. When the rate was increased to 3.0 per cent, definite toxic effects were observed. Numerous investigations have indicated that benzoic acid in the animal organism is detoxified by combining with glycine to form hippuric acid. It has been suggested that the animal organism has a readily available supply of glycine which can be used for the detoxication of benzoic acid. This study indicates that the animal organism has only a limited capacity for detoxifying benzoic acid in the body. The fact that addition of glycine to the sodium benzoate diet increased the amount of gain indicates a decrease in toxicity. The small difference between the gains produced in this group and the control group is considered significant, however, because individual comparisons showed that the control animals made greater gains than the experimental animals in every case.

The animal organism may develop a tolerance for sodium benzoate. Three groups of rats, which had received, respectively, no sodium benzoate, 1.0 per cent sodium benzoate, and 3.0 per cent sodium benzoate plus glycine, were all placed on a diet containing 3.0 per cent sodium benzoate. The group which had not previously received any sodium benzoate made the smallest gain and required the most food per gram of gain. The development of a tolerance for sodium benzoate was indicated by this fact and by the evidence obtained in series II that the toxic effects are expressed during the first 20 days of feeding.

The paired-feeding method of controlling food intake is believed essential to a study of this nature. If the food intakes of the control and experimental animals had not been equal, observed differences in amount of gain could have resulted from the difference in amount of food consumed.

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DISTRIBUTION OF DIACETYL AND ACETYLMETHYLCARBINOL BETWEEN FAT AND WATER, WITH SPECIAL REFERENCE TO BUTTER[1]

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In butter made with butter culture, diacetyl and acetylmethylcarbinol are derived largely from the culture added to the cream and from the fermentation of citric acid during holding or ripening of the cream. Only small portions of these compounds present in the cream at churning appear in the finished butter. They are carried out in the buttermilk (2, 4, 5, 6, 8, 9, 15, 16, 17, 19) in relatively large amounts and also may be removed by the water used to wash the butter (6, 11, 13, 17).

Fat and water, the two constituents of butter which are present in large amounts, have very different solvent properties. The data reported herein deal with the distribution of diacetyl and acetylmethylcarbinol between fat and water and involve studies on (a) mixtures of "Wesson" oil and water or brine, (b) mixtures of butterfat and water or brine and (c) unsalted and salted butter.

ANALYTICAL PROCEDURES

Diacetyl was determined by the colorimetric method of Prill and Hammer (18), with minor modifications used by Hoecker and Hammer (9). Color intensities were obtained with a Klett-Summerson photoelectric colorimeter. Acetylmethylcarbinol also was determined colorimetrically, using the procedure described by Hoecker and Hammer (9) for its estimation in butter.

EXPERIMENTAL

Distribution of Diacetyl and Acetylmethylcarbinol in Mixtures of "Wesson" Oil and Water or Brine[2]

The distribution of diacetyl between "Wesson" oil and water or brine was studied (a) by adding various amounts of an oil solution of diacetyl to 100 ml. portions of oil and then adding 100 ml. of either water or brine to each portion and (b) by adding various amounts of a water solution of diacetyl to 100 ml. portions of either water or brine and then adding 100 ml. of oil to each portion. The mixtures were held 2 days at room temperature in closed containers, with frequent shaking. The oil and water or brine were separated by centrifuging, the oil was pipetted off and diacetyl contents of

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[2] Brine regularly consisted of 14 gm. sodium chloride made up to 100 ml. with distilled water.

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CONVERSION OF PROPIONATE TO LIVER GLYCOGEN IN THE INTACT RAT, STUDIED WITH ISOTOPIC PROPIONATE*

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It has been found that when various fatty acids containing an odd number of carbon atoms in the chain (C_3, C_5, C_7, C_9 acids) are fed to fasted rats deposition of liver glycogen occurs, which is of approximately the same magnitude for each acid (4). The feeding of valeric, heptylic, pelargonic, and undecyclic acids to well fed rabbits leads to an increase in blood acetone bodies, while the administration of propionic acid does not (5). Similarly, incubation of the C_5, C_7, and C_9 acids with liver slices has been found to result in acetoacetate formation, which did not occur when propionate was used as the substrate (6). On the basis of these findings the odd carbon fatty acids have been considered as undergoing successive β oxidation, with the formation of propionate from the terminal 3-carbon residue. Recently Atchley (7) has demonstrated the formation of propionate in a kidney enzyme preparation, using valerate as the substrate. The fate of propionate itself in the animal body, however, is far from established. Propionate given to phlorhizinized dogs (8) and fasted rats (4) leads to the excretion of extra glucose and the deposition of liver glycogen, respectively. While these findings may be accepted as evidence for the conversion of propionate to carbohydrate, the chemical pathway for this transformation remains to be defined.

$$\underset{\text{Propionate}}{CH_3\cdot CH_2\cdot COO^-} \xrightarrow{-2H} \underset{\text{Acrylate}}{CH_2{:}CH\cdot COO^-} \xrightarrow{+HOH} \underset{\text{Lactate}}{CH_3\cdot CHOH\cdot COO^-} \xrightarrow{-2H} \underset{\text{Pyruvate}}{CH_3\cdot CO\cdot COO^-}$$

* This work was supported in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, and by a grant to the Department of Biochemistry from the Prentiss Foundation. Preliminary reports of some parts of this work have appeared (1–3). The radiocarbon used in this work was obtained on allocation from the United States Atomic Energy Commission.

Hahn and Haarmann (9) have reported the accumulation of pyruvate in a muscle mince incubated with propionate and have suggested the accompanying series of reactions to account for their finding.

Bloch and Rittenberg (10), using deuterium as a labeling agent, have examined the ability of a variety of compounds to acetylate fed foreign amines. Propionate and alanine were studied, the latter being considered equivalent to pyruvate metabolically. On the basis of the deuterium content of the excreted acetyl groups, alanine was judged to be an efficient acetylating agent, while propionate was not. This was considered as evidence against the conversion of priopionate to pyruvate.

The present study, with use of carbon-labeled propionate, was undertaken in an effort to gain more information concerning the fate of propionate in the animal body. Variously labeled isotopic propionates, plus glucose, were fed to fasted rats, and, after a suitable period of time, the livers were removed, the glycogen isolated and hydrolyzed to glucose, and the distribution of isotope in the glucose chain determined. Although the observed isotope distribution patterns have been found to bear a basic resemblance to those obtained with similarly labeled lactate (11), a finding pointing to pyruvate as an intermediate in propionate metabolism, there are certain differences between the two sets of patterns which indicate that a direct conversion to pyruvate or lactate may not occur.

Methods

The following compounds were studied: $CH_3 \cdot CH_2 \cdot C^{13}OONa$, $C^{13}H_3 \cdot CH_2 \cdot COONa$, and $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$. The carboxyl-labeled compound was synthesized by the Grignard reaction. The β-labeled propionate was prepared from $C^{13}H_3 \cdot COOH$ (3), and the C^{14},C^{13}-labeled compound from $C^{14}H_3 \cdot C^{13}OOH$ (11). The labeled acetates were reduced to ethanol by catalytic hydrogenation, then converted to ethyl iodide, and the corresponding propionate prepared via nitrile formation and hydrolysis (11). The partition coefficient (12) was determined for each of the three isotopic propionates and agreed with established values within experimental error. Conversion of the C^{14},C^{13}-propionate to lactate, and stepwise degradation of the lactate by well established procedures, showed the isotope to be distributed as anticipated from the synthetic methods employed (11).

The general plan of the experiments and the methods employed have been fully described in prior communications (13, 14, 11). In the experiments with $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$, the glucose from the liver glycogen was degraded by the two types of procedure previously described (13).

RESULTS AND DISCUSSION

The general experimental data are summarized in Table I. In Table II are presented the results of the glycogen degradations. The data from

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DISCUSSION

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the experiments with $C^{13}H_3 \cdot CH_2 \cdot COONa$ and $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$, when consideration is given to the known errors in the degradation methods (14), show that isotope originating in the α- and β-carbon of propionate is completely randomized between the 1,6 and 2,5 positions of glucose. The errors in the present degradation methods, as pointed out previously (14), are that fraction 1,6 is usually subject to contamination with a small amount of non-isotopic carbon, and that the fraction for carbon 3 is not entirely free of carbon from other positions. Carbon 6, from the methyl glucoside degradation, is obtained by a series of fairly specific reactions (13), and appears to be free from such contamination. On the assump-

TABLE I

General Experimental Data from Rats

Experiment No.	Body weight after fast	Sex	Type of propionate administered	Amount of propionate administered per 100 gm. body weight	Amount of glucose administered per 100 gm. body weight	Liver weight	Liver glycogen (as glucose)
	gm.			*mM*	*mM*	*gm.*	*mM*
1	240	♂	$CH_3 \cdot CH_2 \cdot C^{13}OONa$	2.60	2.32	7.2	0.19
2	210	♂	"	3.00	2.65	6.3	0.27
3	196	♀	$C^{13}H_3 \cdot CH_2 \cdot COONa$	2.12	3.53	4.6	0.56
4	182	♀	"	2.10	3.50	5.7	
5	188	♀	"	2.10	3.50	6.0	0.52
6	197	♀	"	2.11	3.51	6.7	0.23
7	210	♂	$C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$	2.51	3.32	6.6	5.19
8	230	♂	"	2.54	3.37	7.0	
9	210	♂	"	2.54	3.37	7.3	
10	240	♂	"	2.66	3.52	8.4	
11	190	♂	"	2.70	3.54	6.6	3.98
12	220	♂	"	2.66	3.52	7.0	
13	230	♂	"	1.92	2.55	7.2	

tion that the hexose chain is formed from 2 triose molecules of similar isotopic composition, carbon 6 may be taken as a measure of the isotope content of fraction 1,6. It will be noted that, in Experiments 7 to 10 and 11 to 13, both the C^{13} and C^{14} values for carbon 6 are in excellent agreement with the values obtained for carbons 2,5. Likewise there is good agreement between the experimentally determined isotope concentrations in the carbon 1, 2, 4, and 5 fraction of the methyl glucoside degradation and the values calculated for this fraction (*cf.* Table II). The over-all results are therefore taken to indicate that the isotope concentrations in the 1, 2, 5, and 6 positions are equal.

Calculations of the per cent of administered isotope recovered in the

1, 2, 5, and 6 carbons of the glucose, presented in parentheses in the last column of Table II, yield values for C^{13} and C^{14} which are identical within experimental error, as might be expected for a mechanism of conversion of propionate to glucose which involved complete randomization of the α- and β-carbons of the propionate between the 1,6 and 2,5 carbons of the glucose.

TABLE II

Distribution of Isotope in Liver Glycogen after Administration of Labeled Propionate to Rats*

Experiment No.	Type of propionate administered	Type of isotope	Isotope concentration in labeled position of administered propionate	Isotope concentration in degradation fractions of glucose: Carbon atoms of glucose 3,4	2,5	1,6	Isotope concentration in degradation fractions of methyl glucoside: Carbon atoms of glucose 3	1,2,4,5	6	Per cent of administered isotope recovered in liver glycogen
1	$CH_3 \cdot CH_2 \cdot C^{13}OONa$	C^{13}	4.62	0.15	−0.01					0.2
2	"	"	4.62	0.12	0.01					0.2
3–6	$C^{13}H_3 \cdot CH_2 \cdot COONa$	"	2.28	0.04	0.17	0.16				2.64
7–10	$C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$	C^{13}	6.50	0.09	0.38	0.35	0.09	0.32 (0.31)†	0.39	5.93 (5.31)‡
		C^{14}	11,600	183	683	645	198	548 (558)†	691	6.07 (5.35)‡
11–13	"	C^{13}	6.50	0.08	0.41	0.37	0.09	0.32 (0.33)†	0.41	7.17 (6.54)‡
		C^{14}	11,600	183	748	642	223	607 (607)†	746	7.50 (6.68)‡

* The C^{13} values are given in atom per cent excess, and the C^{14} values in counts per minute per mg. of carbon.

† The figures in parentheses are calculated values, in which the isotope concentration for carbon 4 is taken as equal to fraction 3,4 and that in carbons 1, 2, and 5 is taken as equal to fraction 2,5.

‡ The values in parentheses represent the per cent of administered isotope recovered in carbons 1, 2, 5, and 6, the isotope values for the 2,5 fraction being taken as representative of all 4 carbon atoms.

As previously reported (11), when $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ is fed to a fasted rat, the α- and β-carbons of the lactate are largely, though not completely, randomized between the 1,6 and 2,5 carbons of the glucose from the resulting glycogen. The distribution is, in general, the same as that noted in the present experiments with $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$, except that in the case of $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ C^{13} is present in somewhat higher concentration in carbons 2,5 than in carbons 1,6, while C^{14} is more abundant in the latter fraction than in the former. The incomplete

ose, presented in parentheses in the last
or C^{13} and C^{14} which are identical within
expected for a mechanism of conversion
nvolved complete randomization of the
ate between the 1,6 and 2,5 carbons of

TABLE II

pen after Administration of Labeled Propionate to Rats

	Isotope concentration in degradation fractions of glucose			Isotope concentration in degradation fractions of methyl glucoside			Per cent of administered isotope recovered in liver glycogen
	Carbon atoms of glucose			Carbon atoms of glucose			
	3,4	2,5	1,6	3	1,2,4,5	6	
2	0.15	−0.01					0.2
2	0.12	0.01					0.2
3	C	1.17	0.16				2.64
0	0.	38	0.35	0.09	0.32 (0.31)†	0.39	5.93 (5.31)‡
00	18.	.83	645	198	548 (558)†	691	6.07 (5.35)‡
0	0.08	0.41	0.37	0.09	0.32 (0.33)†	0.41	7.17 (6.54)‡
00	183	748	642	223	607 (607)†	746	7.50 (6.68)‡

per cent excess, and the C^{14} values in counts

iculated values, in which the isotope concen-
to fraction 3,4 and that in carbons 1, 2, and 5

ent the per cent of administered isotope re-
isotope values for the 2,5 fraction being taken
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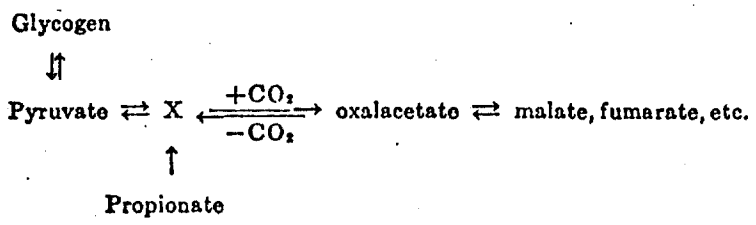
.5 than in carbons 1,6, while C^{14} is more
than in the former. The incomplete

randomization, in the case of lactate, has been explained in terms of the reactions of glycolysis and the tricarboxylic acid cycle. The fact that complete equalization does not occur has been ascribed to conversion to pyruvate and direct incorporation of some of the pyruvate into glycogen, prior to randomization of the isotope via the tricarboxylic acid cycle (11).

If one assumes that pyruvate is an obligatory precursor of glucose and glycogen, the isotope distribution patterns observed in the glycogen in the present studies indicate that propionate labeled in the α- or β-carbon gives rise to pyruvate in which both α- and β-carbons contain equal labeling.

In view of the results with labeled lactate (11), this may be taken to mean that propionate cannot be converted more or less directly to pyruvate (as, for example, via acrylate and lactate (9)), but that a more complex mechanism involving complete randomization of the α- and β-carbons intervenes. What this mechanism might be is a matter of conjecture. One possibility may be indicated in simplified form. Carboxylation on the methyl group of propionate would lead to symmetrical succinate. From such succinate (via fumarate, malate, and oxalacetate), pyruvate would be formed containing isotope from the α or β position of propionate equally distributed between both the α- and β-carbons. However, the authors are aware of no evidence for such a reaction in mammalian tissues. The reverse reaction, the formation of CO_2 and propionate by the decarboxylation of succinate, has been described in certain microorganisms (15–17). It is not meant to suggest that the statement, succinate $\rightarrow$ propionate + CO_2, describes anything but the net result of what may be a series of reactions. Similarly, it is intended only to point out that an over-all conversion of propionate to succinate by a $C_1 + C_3$ addition would account for the isotopic data (as well as for the glycogenic properties of propionate).

On the other hand, a more direct path from propionate to pyruvate may exist, but, because of differences in reaction rates, the major part of the propionate carbon traverses an indirect pathway to pyruvate, involving randomization of isotope. A hypothetical example is presented in the accompanying diagram.



Propionate is depicted as being converted without randomization to an intermediate, X, lying between pyruvate and the reactions of the tricarboxylic acid cycle. If the reaction rate from X toward the randomizing reactions of the tricarboxylic cycle is sufficiently greater than that from X toward pyruvate, isotope from the α- or β-carbons of propionate could be fairly completely randomized between the α and β positions of the pyruvate formed from X. The only available evidence bearing on this suggestion is the finding by Utter and Chenoweth (18) that pyruvate may not be the immediate reactant involved in the CO_2 fixation reaction by which oxalacetate is formed.

TABLE III

Data on Respiratory CO_2 of Rats

Experiment No.	Type of propionate administered	CO_2 output in mM*			Type of isotope	Isotope concentration† in respiratory CO_2			Per cent of administered isotope recovered in respiratory CO_2
		1st period	2nd period	3rd period		1st period	2nd period	3rd period	
1	$CH_3 \cdot CH_2 \cdot C^{13}OONa$	8.7	8.5	7.9	C^{13}	0.12	0.21	0.23	16.6
2	"	7.2	6.2	6.3	"	0.13	0.25	0.27	13.1
3–4	$C^{13}H_3 \cdot CH_2 \cdot COONa$	12.4	12.2	14.8	"	0.08	0.08	0.10	19.3
5–6	"	13.7	14.2	13.9	"	0.06	0.09	0.08	17.4
7	$C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$	8.9	8.5	8.6	"	0.12	0.22	0.26	15.2
8	"	7.5	8.5	8.6	C^{14}	232	473	518	17.3
					C^{13}	0.13	0.17	0.07	7.9
					C^{14}	218	369	188	9.4

* The CO_2 collection periods were 50 minutes, except for Experiments 7 and 8, in which they were 1 hour.

† See foot-note to Table II.

It is interesting to note that the degree of isotope dilution undergone by the $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$ in its conversion to liver glycogen is of the same order as that found for $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ (11). This together with the observed isotope distribution patterns indicates that propionate carbon gives rise to pyruvate (assuming pyruvate to be an obligatory intermediate in glucose and glycogen formation), and at about the same rate as lactate. That the findings of Bloch and Rittenberg using deuterium-labeled propionate in isotope dilution experiments (10), as pointed out earlier, are not in agreement with the foregoing conclusion may have been due to the loss of deuterium from the propionate in the same reactions which bring about the complete randomization of the α- and β-carbons of propionate in the 1, 2, 5, and 6 positions of the glucose in the present experiments.

onverted without randomization to an ruvate and the reactions of the tricar- rate from X toward the randomizing is sufficiently greater than that from ne α- or β-carbons of propionate could ween the α and β positions of the pyru- vailable evidence bearing on this sug- Chenoweth (18) that pyruvate may not in the CO_2 fixation reaction by which

LE III
ratory CO_2 of Rats

utput me*		Type of isotope	Isotope concentration† in respiratory CO_2			Per cent of administered isotope recovered in respiratory CO_2
nd period	3rd period		1st period	2nd period	3rd period	
.5		C^{13}	0.12	0.21	0.23	16.6
.2		"	0.13	0.25	0.27	13.1
.2	14.8	"	0.08	0.08	0.10	19.3
.2	13.9	"	0.06	0.09	0.08	17.4
.5	8.6	"	0.12	0.22	0.26	15.2
		C^{14}	232	473	518	17.3
.5	8.6	C^{13}	0.13	0.17	0.07	7.9
		C^{14}	218	369	188	9.4

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degree of isotope dilution undergone conversion to liver glycogen is of the $H_3 \cdot C^{13}HOH \cdot COONa$ (11). This to- distribution patterns indicates that ruvate (assuming pyruvate to be an and glycogen formation), and at about ne findings of Bloch and Rittenberg in isotope dilution experiments (10), greement with the foregoing conclusion deuterium from the propionate in the the complete randomization of the α- 1, 2, 5, and 6 positions of the glucose

Some of the other experimental results obtained in the present study are worthy of note. The data for respiratory CO_2 in Experiments 7 and 8 (Table III) indicate that the α- and β-carbons of propionate are converted to CO_2 at approximately equal rates. Although the differences between the rate of recovery of C^{13} and C^{14} in the respiratory CO_2 appear fairly large, they are not significant because of the relatively larger error of the isotopic measurements on the respiratory CO_2 samples, owing particularly to the low C^{13} levels in the respiratory CO_2. It may be noted, incidentally, that the glycogen degradation data in the experiments with $CH_3 \cdot CH_2 \cdot C^{13}OONa$ and $C^{13}H_3 \cdot CH_2 \cdot COONa$ rule out β oxidation to malonate with the formation of acetate (or an equivalent 2-carbon compound) from the α- and carboxyl carbons of malonate as an important pathway in propionate metabolism. Since both types of propionate would yield carboxyl-labeled malonate, and consequently a carboxyl-labeled 2-carbon compound, this mechanism should deposit isotope exclusively in positions 3 and 4 of the glucose from liver glycogen following feeding of both types of propionate (14). The finding of label predominantly in the 1,6 and 2,5 carbons in the experiments with the β-labeled propionate cannot be reconciled with this pathway.

It should be clear that the foregoing discussion cannot be generalized to extrahepatic tissues, in which reactions may occur that will not affect the isotope distribution in liver glycogen. In this connection it should be pointed out that in many instances in the present and in former experiments with other labeled compounds glycogen of cardiac and skeletal muscles has been isolated and degraded. Isotope concentrations have always been much lower than those found in liver glycogen, but the distribution patterns have always been the same. While this may indicate basically similar metabolic pathways in liver and muscle for the compounds concerned, it may also be nothing more than a reflection of hepatic metabolism via the blood sugar.

SUMMARY

Isotopic propionate ($CH_3 \cdot CH_2 \cdot C^{13}OONa$, $C^{13}H_3 \cdot CH_2 \cdot COONa$, and $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$) has been fed to fasted rats and the resulting liver glycogen isolated, hydrolyzed to glucose, and degraded. Isotope from the carboxyl group of propionate has been found to give detectable labeling only in carbon atoms 3 and 4 of the glucose, while propionate containing isotope in the α or β position gives rise to glucose labeled predominantly and equally in carbons 1, 2, 5, and 6.

If it is assumed that pyruvate is an obligatory intermediate in glucose and glycogen formation, the results indicate that propionate gives rise to pyruvate, but that in the process the α- and β-carbons of the propionate

lose their orientation and become equally distributed between the corresponding groups of pyruvate. Some possible explanations for this finding are advanced.

It is a pleasure to acknowledge the help of Dr. Walton W. Shreeve in several of the experiments.

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Utilization of propionate for heme synthesis in human bone marrow cells in vitro

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The incorporation of ^{14}C-propionate into heme in vitro was measured in normal and megaloblastic bone marrow cells. In megaloblastic bone marrow cells obtained from patients with vitamin B_{12} deficiency, the incorporation of ^{14}C-propionate into heme was markedly reduced. An addition of coenzyme B_{12}—dimethylbenzimidazole cobamide coenzyme B_{12}—into the incubation medium resulted in an apparent increase in the amount of ^{14}C-propionate incorporated into heme. In megaloblastic or megaloblastoid bone marrow cells obtained from patients without vitamin B_{12} deficiency, an increase in ^{14}C-propionate incorporated by adding coenzyme B_{12} was not observed. The pathophysiological and clinical significance of these findings is discussed in relation to changes in propionate metabolism in vitamin B_{12}-deficient states.

The existence of the pathway from propionyl-CoA to methylmalonyl-CoA and the requirement of B_{12} coenzyme for the subsequent conversion of methylmalonyl-CoA to succinyl-CoA have been well established in several organs.[1-5] The succinyl-CoA has been a known precursor of heme since the work of Gibson and associates,[6, 7] and Kikuchi and associates.[8]

We have presumed that if the pathway from propionyl-CoA to succinyl-CoA exists in human immature erythroid cells, then the propionate would be incorporated into heme in these cells, and that the incorporation would be impaired in vitamin B_{12} deficiency.

The present study has been undertaken with the purpose of demonstrating the utilization of propionate for heme synthesis in human bone marrow cells. Normal and megaloblastic bone marrow cells were compared in vitro with respect to both the incorporation of ^{14}C-propionate into heme and the effect of cobamide coenzyme B_{12} on this process.

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Materials and methods

Case studies. Laboratory data of the cases studied in this report are shown in Tables I and II. In all cases in Table I, the existence of vitamin B_{12} deficiency was confirmed with 2 or more positive results among the following tests: reduced absorption of labeled vitamin B_{12} (Schilling test), good hematological response to vitamin B_{12} therapy, low serum vitamin B_{12} level, and increased urinary excretion of methylmalonic acid. All cases in Table II revealed megaloblastic or megaloblastoid bone marrow changes. However, in these cases, laboratory data did not indicate the presence of vitamin B_{12} deficiency. Their serum vitamin B_{12} levels were within normal limits. Absorption of labeled B_{12} was normal in all cases. Megaloblasts in cases of erythroleukemia had a positive PAS stain. These patients with erythroleukemia eventually died of myeloblastic leukemia. In the sideroblastic anemia case, typical ringed sideroblasts were observed in about 60 per cent of the immature erythroid cells. In this case and in the case of erythroleukemia (Case No. 3 in Table II), urinary excretion of methylmalonic acid was within normal limits. In the cases of sideroblastic anemia and benzol intoxication, bone marrow erythroblasts had nuclear patterns reminiscent of megaloblasts, megaloblastoid erythroblasts. Typical megaloblasts, however, were not observed in these cases.

Bone marrow cell incubation. The heparinized bone marrow aspirate obtained by sternal puncture was suspended in a medium consisting of equal parts of NCTC 199 and autologous plasma with a final cellular concentration of 10^7 nucleated cells per milliliter. The medium also contained 40 units of penicillin-G and 0.05 μmoles of sodium propionate-2-^{14}C (10 mc. per millimole, purity 99 per cent, The Radiochemical Center, England) per milliliter. When the incorporation of ^{14}C-glycine into heme was measured in vitro, the bone marrow cells were suspended in a solution containing sodium chloride 130 μmoles, potassium chloride 5.2 μmoles, magnesium chloride 7.4 μmoles, glucose 5.6 μmoles, potassium phosphate buffer 20 μmoles, and 0.047 μmoles of glycine-2-^{14}C (21.5 mc. per millimole, purity more than 99 per cent) per milliliter. Incubation tubes containing 1 ml. of the bone marrow cell suspension were incubated in duplicate at 37° C. in a CO_2 gas-incubator containing 5 per cent CO_2 and 95 per cent air. In each series of incubation, a zero time sample was prepared. In those cases in which the incorporation of ^{14}C-glycine into heme was studied, each incubation tube contained 0.5 ml. of the incubation mixture. When the medium contained dimethylbenzimidazole cobamide coenzyme B_{12} (Yamanouchi Pharmaceutical Company, Japan) at a concentration of 10 μg per milliliter, care was taken to avoid light throughout the preparation and the incubation of the cell suspension. Numbers of the cells in the suspension were counted, and the hemoglobin concentration was measured colorimetrically as a cyanmethemoglobin with Acuglobin (Ortho Diagnostics) as the standard. Smears prepared from the cell suspension were stained with Wright's stain, and the percentage of the erythroblasts was determined by counting 500 nucleated cells. After incubation, the cells were washed three times with cold saline, and 100 mg. of carrier hemoglobin was added per tube.

Preparation of crystalline hemin. Crystalline hemin was prepared by the method of Chu and Chu[9] with a modification. Five milliliters of a mixture of acetone and glacial acetic acid (4:1) was added to the tube containing washed cells and carrier hemoglobin. After thorough mixing, the tube was centrifuged for 5 minutes at 2,000 r.p.m. The same procedures were repeated three more times, and the supernatant fluid was collected into a test tube. About 10 mg. of sodium chloride was added per tube. Acetone was removed from the supernatant fluid by heating at about 70° C. for several hours. In the meantime, crystalline hemin was formed. The hemin crystal was washed four times with distilled water and dissolved into 1.5 ml. of 5.9M ammonia water. One milliliter of the hemin solution was plated on a planchette and dried. The radioactivity of the hemin was counted with a gas-flow counter. Counting efficiency of the labeled hemin was 40 per cent, and preliminary studies revealed that the amounts of hemin plated did not affect the counting efficiency. Few drops of 1.54M potassium cyanide was added to 0.2 ml. of the hemin solution. After diluting to 20 ml. with water, the amount of cyanhematin was measured colorimetrically with a Hitachi spectrophotometer at 545 mμ. A standard curve of cyanhematin was obtained by dissolving the crystalline hemin (Daiichi Pure Chemicals, Japan) into 5.9M ammonia

Table I. Hematological data of the cases with B_{12} deficiency

Case No.	*Patient*	*Sex*	*Age (years)*	*Diagnosis*	*R.B.C. × 10⁴*	*Hgb. Gm./dl*
1	K. S.	Male	72	Pernicious anemia	216	10.3
2	J. N.	Male	68	Pernicious anemia	64	2.8
3	N. Y.	Female	52	Pernicious anemia	93	4.0
4	Y. I.	Male	60	Pernicious anemia	213	9.2
5	I. W.	Male	39	Blind loop	203	8.1
6	Y. N.	Male	33	Blind loop	137	7.4
7	K. S.	Male	34	Total gastrectomy	144	6.6

*C.I. = color index.

Table II. Hematological data of the cases without B_{12} deficiency

No.	*Patient*	*Sex*	*Age (years)*	*Diagnosis*	*R.B.C. × 10⁴*	*Hgb. Gm./dl*
1	S. I.	Male	43	Erythroleukemia	193	7.1
2	Y. Y.	Male	27	Erythroleukemia	171	6.6
3	F. S.	Female	54	Sideroblast anemia	229	9.4
4	T. H.	Male	39	Benzol intoxication	215	9.6

water and by measuring the amounts as described. The yield of the crystalline hemin from the bone marrow cells mixed with carrier hemoglobin was about 30 per cent. Amounts of ^{14}C-propionate or ^{14}C-glycine incorporated into heme were calculated from the yield of crystalline hemin and the specific activities of these labeled precursors. All the incubations were performed in duplicate, and the samples with a variance of more than 10 per cent in the duplicate were discarded. They were then expressed as moles of propionate or glycine incorporated into heme per 10^6 erythroblasts.

Results

The rate of ^{14}C-propionate incorporation into heme in normal bone marrow cells was approximately 5 $\mu\mu$moles per 10^6 erythroblasts per hour and was linear over the 6 hour interval studied. Fig. 1 shows an incorporation of ^{14}C-propionate into heme in bone marrow cells of megaloblastic or megaloblastoid anemia. The percentages of megaloblasts in bone marrow cells are shown in Tables I and II.

Hgb. Gm./dl	*C. I.**	*% of megaloblasts in bone marrow*	*Serum B_{12} mμg/ml.*	*Schilling test*	*Urinary MMA mg./day*	*Response to B_{12}*
10.3	1.5	11.5	0.12	+		Good
2.8	1.4	41.5	0.05	+		Good
4.0	1.4	40.5		+		Good
9.2	1.4	10.0		+		Good
8.1	1.2	10.0		+		Good
7.4	1.1	15.0	Not measurable	+	50.0	Good
6.6	1.4	36.5	0.05	+	135.0	Good
			Normal (10.3—0.6)		Normal <2.0	

Hgb. Gm./dl	*Bone marrow*	*Serum B_{12} mμg/ml.*	*Schilling test*	*Urinary MMA (mg./day)*	*Response to B_{12}*
7.1	Myeloblastosis, megaloblasts 16%	4.98	(–)		(–)
6.6	Myeloblastosis, megaloblasts 12%		(–)	Not detectable	(–)
9.4	Ringed sideroblasts, megaloid erythroblastosis	0.49	(–)	1.0	(–)
9.6	Megaloid erythroblastosis	Normal (0.3—0.6)		Normal <2.0	(–)

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The mean value of propionate incorporated into heme was markedly reduced in vitamin B_{12} deficiency. After the treatment with vitamin B_{12}, although the number of patients is few, an inclination to restore the propionate incorporation was observed. In 2 cases of erythroleukemia with megaloblastic bone marrow, the propionate incorporation was decreased. In a case of sideroblastic anemia, the decrease in the incorporation was slight, and in a case of benzol intoxication, the incorporation was within normal limits.

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As shown in Fig. 2, the addition of cobamide coenzyme B_{12} in vitro to the bone marrow cell suspension of vitamin B_{12}–deficient patients resulted in an apparent increase in the incorporation of the propionate into heme. Although the degree of the increase was different in each case, this in vitro response to cobamide coenzyme was observed in all vitamin B_{12}–deficient bone marrow cells. In the megaloblastic or megaloblastoid bone marrow cells obtained from patients

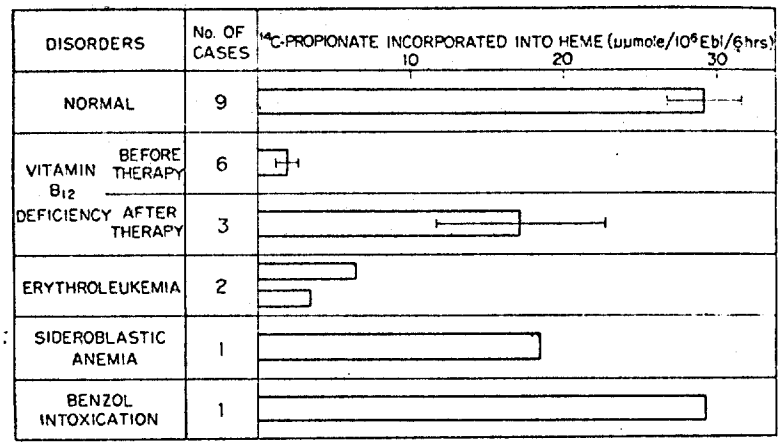

Fig. 1. ^{14}C-propionate incorporation into heme in megaloblastic or megaloblastoid bone marrow cells. The indicated variances are the standard errors. Each bar in columns *2* to *6* is the mean value of duplicate incubation. *Ebl* represents erythroblasts.

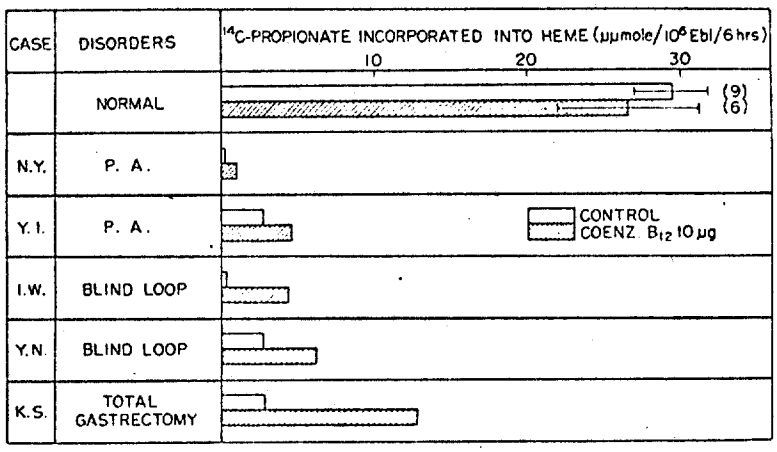

Fig. 2. Effect of coenzyme B_{12} on ^{14}C-propionate incorporation into heme vitamin B_{12} deficient-megaloblastic bone marrow cells. Values in parentheses in column *1* are numbers of the patients studied. The indicated variances are the standard errors. Each bar in columns *2* to *6* is the mean value of duplicate incubation. Open bars represent the control incubations and hatched bars the incubations with coenzyme B_{12} 10 μg per milliliter. *Ebl* represents erythroblasts.

who had no vitamin B_{12} deficiency, the increased propionate incorporation caused by adding cobamide coenzyme B_{12} in vitro was not demonstrated (Fig. 3). When the vitamin B_{12} deficient-megaloblastic bone marrow cells (Case No. 3 in Table I) were incubated with ^{14}C-glycine and the incorporation of the labeled precursor into heme was measured, an addition of cobamide coenzyme did not increase the amounts of incorporation. Similar findings were observed in another vitamin B_{12}-deficient case (Case No. 1 in Table I). The bone marrow

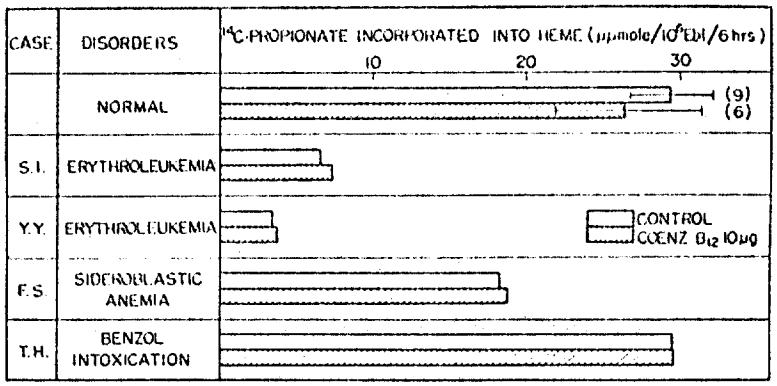

Fig. 5. Effect of coenzyme B_{12} on ^{14}C-propionate incorporation into heme in megaloblastic or megaloblastoid bone marrow cells without B_{12} deficiency. Values in parentheses in column *1* are numbers of the patients studied. The indicated variances are the standard errors. Each bar in columns *2* to *5* is the mean value of duplicate incubation. Open bars represent the control incubations and hatched bars the incubations with coenzyme B_{12} 10 μg per milliliter. *Ebl* represents erythroblasts.

cells of these cases, however, increased the propionate incorporation into heme in response to added cobamide coenzyme B_{12}.

Discussion

In the present in vitro studies, we demonstrated that the propionate was used for heme synthesis in human bone marrow cells. This utilization of propionate for heme synthesis in human bone marrow cells is considered to occur via methylmalonyl-CoA, succinyl-CoA, and delta-aminolevulinic acid. Since urinary excretion of methylmalonic acid is markedly increased in pernicious anemia, the methylmalonyl-CoA mutase, which catalizes the conversion of methylmalonyl-CoA to succinyl-CoA and requires cobamide coenzyme B_{12}, is considered to be deficient in this disorder.[10] Therefore, the present findings on the reduced incorporation of ^{14}C-propionate into heme are considered to suggest an existence of the same disturbance of propionate metabolism at the level of methylmalonyl-CoA mutase in erythroid cells of vitamin B_{12}–deficient patients. These suggestions are also supported by the results of the present studies shown in Fig. 2, in which the addition of coenzyme B_{12} increased propionate incorporation into heme by bone marrow cells from untreated vitamin B_{12}–deficient patients and not by cells from patients with other disorders.

The incorporation of labeled propionate into heme had occurred without any time lag in normal cells, as well as in vitamin B_{12} deficient–bone marrow cells. This absence of a time lag in the utilization of propionate for heme synthesis is considered to suggest the insignificant amount of propionate in human bone marrow cells in normal as well as in pathological states.

Therefore, it is suggested that the difference in the utilization of the labeled propionate for heme synthesis between normal and vitamin B_{12} deficient–bone marrow cells does not represent the difference in the amount of propionate be-

tween these states. It is possible that the present reduction and partial restoration by coenzyme B_{12} of propionate incorporation into heme in vitro in vitamin B_{12} deficient–bone marrow cells could be due to a defect in the heme synthetic process occurring after the condensation of glycine with succinyl-CoA. The inability of coenzyme B_{12} to accelerate the incorporation of ^{14}C-glycine into heme in vitamin B_{12} deficient–bone marrow cells, however, is considered to indicate that the above possibility is less likely. A similar inability of vitamin B_{12} to accelerate in vitro heme synthesis in pernicious anemia bone marrow cells was reported by Thomas and Lochte.[13]

From the results of the present study demonstrating the inability of megaloblastic or megaloblastoid cells without vitamin B_{12} deficiency to respond to coenzyme B_{12}, it is suggested that the present in vitro response to coenzyme B_{12} is specific to vitamin B_{12}–deficient states, and not directly related to morphologically identified megaloblastic changes.

It is noted that in erythroleukemia bone marrow cells with megaloblastic changes, the propionate incorporation was reduced, although the addition of coenzyme B_{12} did not increase the incorporation. Mechanisms, such as the deficient cellular uptake of vitamin B_{12}, folate deficiency, or reduced activities of the enzymes related to heme synthesis, are assumed on the basis of these findings from the megaloblasts of erythroleukemia. However, more information concerning the heme synthesis in bone marrow cells of erythroleukemia will be necessary in order to understand the mechanism involved in our present findings.

The number of moles of propionate utilized for heme synthesis per erythroblast was almost one tenth the number of moles of glycine utilized, indicating that propionate contributes to a small fraction of the succinyl-CoA pool. It is considered, therefore, that the defective utilization of propionate for heme synthesis in vitamin B_{12} deficiency does not mean defective heme synthesis in vitamin B_{12} deficiency, nor does it contribute to the megaloblastic changes. Cellular uptake or incorporation into heme of the radioiron in vitro has been reported to be normal or close to normal in the bone marrow cells from pernicious anemia patients.[11, 12] The methods used in this study to demonstrate the existence of vitamin B_{12} deficiency in bone marrow cells are rather simple. It is considered, therefore, that if our findings can be confirmed by increasing the number of cases studied, the present method of incubating bone marrow cells with ^{14}C-propionate and coenzyme B_{12} might be used as a clinical test to prove the existence of vitamin B_{12}–deficient states.

The authors are grateful to Drs. I. Asai and S. Miwa in Toranomon Hospital, to Dr. T. Maekawa in Gunma University, to Drs. I. Amaki and K. Irimajiri in Nihon University Hospital for the presentation of their cases, to Dr. T. Abe in Tokyo Medical and Dental University for measuring the urinary methylmalonic acid, and to Dr. E. Goldwasser in the University of Chicago for his advice and help in the preparation of this paper.

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Range-Finding Toxicity Data: List VII

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Acute toxicity and irritation data on about 200 compounds are presented, accumulated in a continuing program for screening potential commercial products.

DURING THE SEVEN years since the appearance of our last communication on the range-finding test,[1] many additional data have become available for presentation. Table I lists these data under names conforming to the nomenclature used in *Chemical Abstracts*. Table II lists only the oral LD_{50} values for a number of inorganic compounds. All materials included are either in commercial production or have been evaluated for commercial potential within the past few years.

As has been stated in earlier communications,[1-6] the range-finding test is relied upon only to allow predictions of the comparative hazards of handling new chemicals. Acute toxicity studies, no matter how precisely executed, yield no more than an indication of the degree of care necessary to protect exposed workmen or lead to an opinion that certain technically feasible applications of a chemical may or may not eventually be proved safe.

Our last paper[1] included data under the name of triisooctyl phosphine. The compound should have been entered as triisooctyl phosphite.

Experimental methods have remained unchanged. They are described in our 1962 publication.[1]

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TABLE I
Range-Finding Toxicity Data

Single Oral LD_{50} for Rats	Single Skin Penetration LD_{50} for Rabbits	Concentrated Vapor Inhalation by Rats Maximum for	Inhalation of Metered Vapor Concentration by Rats: Concentration	Inhalation of Metered Vapor Concentration by Rats: Time,	Inhalation of Metered Vapor Concentration by Rats: Mortal-	Irritation on Uncovered Rabbit	Corneal Injury in

List VII

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...e described in our 1962

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Table I
Range-Finding Toxicity Data

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corneal Injury in Rabbits
Hydrocarbons								
2-(4-Cyclohexenyl)-bicyclo-(2.2.1)-hept-5-ene	4.29 (3.07 – 5.98)	3.54 (2.19 – 5.72)	8 hr.	–	–	–	5	2
Cyclopentene	2.14 (1.45 – 3.17)*	1.59 (0.97 – 2.59)	5 m. §	16,000	4	4/6	5	5
5-Ethylidene-2-norbornene	2.83	9.17 (1.35 – 6.33)	–	4,000	4	5/6	5	2
p-Menth-3-ene	20.75 (15.36 –28.03)	5.66 (2.59 –12.3)	30 m.	2,000	4	2/6	5	1
2-Norbornene	11.3 (7.0 –18.3)*	> 5	5 m.	–	–	–	1	7
1,7-Octadiene	19.7 (15.0 –25.8)	14.1 (4.66 –42.9)	15 m.	8,000	4	2/6	4	1
3a,4,7,7a-Tetrahydro-1H-indene	3.73 (2.68 – 5.21)	14.1 (8.75 –22.9)	4 hr.	–	–	–	3	2
Toluene	7.53 (6.73 – 8.43)*	14.1 (9.63 –20.77)	–	4,000	4	1/6	4	9
4-Vinyl-1-cyclohexene	3.08 (2.49 – 3.81)	20	15 m.	8,000	4	4/6	4	2
5-Vinyl-2-norbornene	5.19 (4.22 – 6.41)	15.9 (11.7 –21.5)	–	4,000	4	4/6	5	2
Hydroxyl Compounds								
p-tert. Butylphenol	3.25 (2.48 – 4.26)	2.52 (0.93 – 8.58)	8 hr.	–	–	–	6	9
Cyclohexenyl-4-phenol	2.83 *	3.18 (1.94 – 5.18)*	8 hr.	–	–	–	–	9
2-Cyclopenten-1-ol	0.47 (0.34 – 0.66)	0.18	1 hr.	500†	4	0/6	2	5
3,4-Dihydroxy-1,5-hexadiene	1.62 (1.24 – 2.13)	0.40 (0.29 – 0.54)	–	–	–	–	2	6
Eicosanol, mixed isomers	>64	>20	8 hr.	–	–	–	3	1
2-Ethylhexanol	2.46 (1.82 – 3.33)	2.38 (1.70 – 3.34)	8 hr.	–	–	–	3	5
Hydroxydihydrocyclopentadiene	3.25 (2.11 – 4.99)	3.15 (2.33 – 4.26)	8 hr.	–	–	–	5	9
3-Methyl-1-butanol	7.07 (4.82 –10.4)	3.97 (2.93 – 5.37)	8 hr.	–	–	–	2	8
Methyl-3-cyclohexenyl-methanol	1.41	0.62 (0.42 – 0.92)	8 hr.	–	–	–	3	8
Nonylphenol, mixed isomers	1.62 (1.06 – 2.50)	2.14 (1.52 – 2.99)	4 hr.	–	–	–	6	10
2-Norbornanemethanol	1.62 (1.24 – 2.13)*.	0.80	8 hr.	–	–	–	3	9
Tetradecanol, mixed isomers	32.5 (29.1 –36.3)	7.13 (4.41 –11.52)	8 hr.	–	–	–	2	1
Ethers								
Bicyclo(2.2.1)hept-2,5-ylene bis allyl ether	3.73 (2.43 – 5.74)	5.95 (3.57 – 9.09)	8 hr.	–	–	–	3	1
2-Cyclopentyl ether	11.3	1.59 (1.03 – 2.59)	4 hr.	–	–	–	5	2
Ethyl propenyl ether	19.0 (13.4 –27.0)	>10	5 m.	8,000	4	0/6	2	2
Ethyl vinyl ether	8.16 (7.03 – 9.48)	>20	–	64,000	4	0/6	1	1
Methoxy triethylene glycol vinyl ether	11.3	10.0 (4.56 –21.9)	8 hr.	–	–	–	2	3
alpha-Methylbenzyl ether	9.80 (6.38 –15.06)*	–	8 hr.	–	–	–	4	1
2-Methoxypropene	1.87 (1.26 – 2.76)	>20	–	64,000	4	4/6	1	1
1,1,3,5-Tetramethoxyhexane	4.09 (3.52 – 4.76)*	–	8 hr.	–	–	–	2	2
Hydroxy Ethers								
1-Butoxyethoxy-2-propanol	4.00 (2.55 – 6.27)	2.83 (1.75 – 4.57)	8 hr.	–	–	–	3	5
3-Butoxy-1-propanol	5.95 (3.65 – 9.68)	1.59 (1.17 – 2.15)	8 hr.	–	–	–	2	7
Diethoxytetraethylene glycol	4.29 (2.90 – 6.34)	6.35 (3.89 –10.4)	8 hr.	–	–	–	2	2
3-Ethoxy-1-propanol	13.0 (8.46 –20.0)	2.83	8 hr.	–	–	–	2	5
2-Ethylbutoxypropanol, mixed isomers	3.56 (2.21 – 5.74)	6.0	8 hr.	–	–	–	2	9
2-Hexyloxy-2-ethoxyethyl ether	3.73 (2.68 – 5.21)	6.30 (4.66 – 8.52)	8 hr.	–	–	–	2	5
Isobutoxypropanol, mixed isomers	7.5	3.56 (1.95 – 6.52)	8 hr.	–	–	–	3	7

TABLE I
Range-Finding Toxicity Data (Continued)

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corneal Injury in Rabbits
2-Isopropoxyethanol	5.66 (4.11 – 7.79)	1.60 (1.08 - 2.37)	2 hr.	4,000	4	4/6	3	5
3-Methoxy-1-propanol	13.0 (9.90 –17.0)	5.66	8 hr.	–	–	–	1	5
2-Propoxyethanol	4.89 (4.16 – 5.73)*	0.96 (0.93 - 0.98)	–	–	–	–	2	8
Propoxypropanol, mixed isomers	3.25 (2.48 – 4.26)	4.00 (2.70 - 5.92)	8 hr.	–	–	–	2	7
Aldehydes, Ketones								
Cyclohexanone	1.62 (1.20 – 2.20)	1.00 (0.63 - 1.63)	30 m.	2,000†	4	1/6	1	5
5-Ethyl-3-nonen-2-one	8.12 (7.27 – 9.06)*	8.48 (5.04 -14.24)	8 hr.	–	–	–	2	1
Hexanal, mixed isomers	9.51 (5.84 –15.5)	>10	30 m.	–	–	–	2	5
2-Methylcyclohexanone	2.14 (1.48 – 3.10)	1.77	–	2,800	4	3/6	3	5
2-Methyl-1,2,3,6-tetrahydrobenzaldehyde	5.66	3.15 (2.33 - 4.26)	8 hr.	–	–	–	5	5
1-Tetralone	0.81 (0.62 – 1.07)	>20	8 hr.	–	–	–	4	3
Valeraldehyde	5.66	6	15 m.	4,000	4	3/6	2	5
Acids, Anhydrides, Lactones								
3-Hydroxybutyric acid lactone	17.2 (12.3 –23.9)	>20	8 hr.	–	–	–	5	5
b-Methyl-delta-valerolactone	12.9 (8.4 –19.9)	>10	–	–	–	–	1	4
Methyltetrahydrophthalic anhydride	2.14 (1.48 – 3.10)	1.41	8 hr.	–	–	–	1	9
Tri-isobutenylsuccinic anhydride	0.94 (0.61 – 1.44)*	0.89 (0.55 - 1.44)*	–	–	–	–	4	7
Valeric acid, mixed isomers	2.00 (0.90 – 4.44)	0.31 (0.06 - 1.50)	8 hr.	–	–	–	6	9
Esters, Acetals								
Acetic acid, 2-methyl-2,4-pentanediol diester	3.36 (2.06 – 5.48)	16.0 (3.33 -76.8)	8 hr.	–	–	–	3	2
Acetic acid, 2-methyl-2-propene-1,1-diol diester	0.44 (0.28 – 0.68)	0.044(0.024- 0.082)	1 hr.	62.5	4	5/6	5	9
Acetic acid, phenyl ester	1.63 (1.03 – 2.74)	8.00 (3.65 -17.5)	8 hr.	–	–	–	2	2
Acetic acid, propyl ester	9.8 (7.5 –12.9)	>20	30 m.	8,000	4	4/6	1	2
Acetic acid, triethylene glycol diester	22.6	8.0 (1.91 -33.5)	8 hr.	–	–	–	1	1
Acrylic acid, 2-butoxyethoxy ester	6.50 (4.72 – 8.95)	0.64 (0.39 - 1.04)	8 hr.	–	–	–	5	2
Acrylic acid, decyl ester, mixed isomers	12.3 (9.98 –15.3)	3.54 (1.17 -10.7)	8 hr.	–	–	–	5	1
Acrylic acid, hexyl ester	26.0 (18.9 –35.8)	5.66 (3.50 - 9.14)	4 hr.	–	–	–	5	1
Acrylic acid, 2-hydroxypropyl ester	1.23 (0.89 – 1.70)	0.16 (0.10 - 0.26)	8 hr.	–	–	–	5	7
Acrylic acid, 2-methoxyethoxy ester	0.81 (0.59 – 1.21)	0.25 (0.17 - 0.37)	15 m.	500	4	3/6	4	5
Acrylic acid, 2-norbornyl ester	5.66	0.88 (0.54 - 1.43)	8 hr.	–	–	–	5	2
Azelic acid, dihexyl ester	16.0 (10.2 –25.0)	>20	–	–	–	–	2	1
Benzoic acid, 2-ethylhexanediol diester	29.2 (15.8 –53.9)	20	8 hr.	–	–	–	2	1
Fumaric acid, ethyl 2,3-epoxypropyl ester	1.62 (1.06 – 2.50)	0.35 (0.22 - 0.57)	–	–	–	–	5	8
Fumaric acid, dimethyl ester	2.24 (1.15 – 4.37)*	1.25 (0.84 - 1.85)*	8 hr.	–	–	–	5	9
Malonic acid, diethyl ester	14.9 (10.7 –20.8)	>16	8 hr.	–	–	–	2	5
Methacrylic acid, allyl ester	0.43 (0.31 – 0.60)*	0.50 (0.37 - 0.68)	8 m.	250†	4	0/6	5	2
Methacrylic acid, butyl ester	22.6	11.3 (5.19 -24.7)	8 hr.	–	–	–	5	1
Phthalic acid, butyl decyl ester	20.8 (16.8 –25.7)	15.9 (11.7 -21.5)	8 hr.	–	–	–	2	1
Phthalic acid, decyl hexyl ester	49.4 (39.9 –61.0)	>20	8 hr.	–	–	–	2	1

(Continued on Next Page)

TABLE I
Range-Finding Toxicity Data (Continued)

			Concen-	Inhalation of Metered Vapor Concentration			Irritation	Corneal

Acrylic ac[illegible]yl ester	26.0 (18.9 -35.8)	[illegible]	4 hr.	–	–			
Acrylic acid [illegible]droxypropyl ester	1.23 (0.89 - 1.70)	[illegible]	8 hr.	–	–			
Acrylic acid, 2-methoxyethoxy ester	0.81 (0.59 - 1.21)	[illegible]	15 m.	500	4	3/6	[illegible]	[illegible]
Acrylic acid, 2-norbornyl ester	5.66	[illegible]	8 hr.	–	–	–	5	2
Azelic acid, dihexyl ester	16.0 (10.2 -25.0)	>20		–	–		2	1
Benzoic acid, 2-ethylhexanediol diester	29.2 (15.8 -53.9)	20	8 hr.	–	–	–	2	1
Fumaric acid, ethyl 2,3-epoxypropyl ester	1.62 (1.06 - 2.50)	0.35 (0.22 - 0.57)	–	–	–	–	5	8
Fumaric acid, dimethyl ester	2.24 (1.15 - 4.37)*	1.25 (0.84 - 1.85)*	8 hr.	–	–	–	5	9
Malonic acid, diethyl ester	14.9 (10.7 -20.8)	>16	8 hr.	–	–	–	2	5
Methacrylic acid, allyl ester	0.43 (0.31 - 0.60)*	0.50 (0.37 - 0.68)	8 m.	250†	4	0/6	5	2
Methacrylic acid, butyl ester	22.6	11.3 (5.19 -24.7)	8 hr.	–	–	–	5	1
Phthalic acid, butyl decyl ester	20.8 (16.8 -25.7)	15.9 (11.7 -21.5)	8 hr.	–	–	–	2	1
Phthalic acid, decyl hexyl ester	49.4 (39.9 -61.0)	>20	8 hr.	–	–	–	2	1

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TABLE I

Range-Finding To[illegible]

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corneal Injury in Rabbits
Phthalic acid, decyl octyl ester	45.2 (28.0 -73.2)	>20	8 hr.	–	–	–	2	1
Propionaldehyde dicrotyl acetal	2.14 (1.54 - 2.99)	3.54 (2.19 - 5.72)	30 m.	2,000	4	5/6	4	2
Succinic acid, di-2-[(2-ethylbutoxy)ethoxy] ester	3.3 *	7.11 (6.58 - 7.69)	–	–	–	–	3	2
Epoxy Compounds								
Bicyclononadiene diepoxide	2.14 (1.54 - 2.99)	1.77	8 hr.	–	–	–	3	5
Bis(3,4-epoxybutyl)ether	1.07 (0.77 - 1.50)	0.25 (0.17 - 0.37)	8 hr.	–	–	–	5	7
Bis(3,4-epoxy cyclohexylmethyl)adipate	4.39 (3.07 - 5.98)	>20	8 hr.	–	–	–	2	1
N,N-Bis(2,3-epoxypropyl)aniline	1.62 (1.18 - 2.24)	3.56 (1.68 - 7.57)	8 hr.	–	–	–	3	1
Butyl 2,3-epoxypropyl fumarate	0.71 (0.48 - 1.04)	1.26 (0.93 - 1.70)	8 hr.	–	–	–	5	8
t-Butylphenyl 2,3-epoxypropyl ether	3.73 (2.68 - 5.21)	5.66	8 hr.	–	–	–	5	2
1,2-Epoxy cyclohexane	1.09 (0.66 - 1.80)	0.63 (0.39 - 1.03)	30 m	2,000†	4	1/6	3	8
2,3-Epoxypropyl methacrylate	0.77 (0.57 - 1.04)	0.45 (0.21 - 0.95)	2 hr.	–	–	–	5	4
Propylene oxide	1.14 (1.05 - 1.23)	1.50 (1.07 - 21.0)	5 m. §	4,000	4	4/6	1	5
Heterocyclic Oxygen								
2-Aminomethyl-2,3-dihydro-4H-pyran	1.00 (0.64 - 1.57)*	0.18	4 hr.	–	–	–	6	9
2,5-Divinyltetrahydropyran	2.46 (1.79 - 3.39)	1.41 (0.65 - 3.08)	4 hr.	–	–	–	4	2
3,9-Divinyl-2,4,8,10-tetraoxaspiro (5,5) undecane	3.25 (2.48 - 4.26)*	7.92 (5.87 -10.73)*	8 hr.	–	–	–	2	2
2-Ethoxy-4-methyl-tetrahydropyran	5.16 (4.17 - 6.38)	> 5	4 hr.	–	–	–	2	3
Aliphatic and Alicyclic Amino Compounds								
1-Amino-3-aminomethyl-3,5,5-trimethyl cyclohexanol	1.30 (1.04 - 1.75)	–	8 hr.	–	–	–	7	9
2-Aminomethyltetrahydropyran	0.71 (0.44 - 1.13)	0.71 (0.44 - 1.14)	4 hr.	–	–	–	6	9
3-Aminopropoxy-2-ethoxy ethanol	6.50 (4.95 - 8.53)*	5.99 (4.27 - 8.42)	8 hr.	–	–	–	3	7
Amyl amine, mixed isomers	0.47 (0.33 - 0.65)*	1.12 (0.61 - 2.05)	30 m. §	2,000	4	4/6	6	9
Bis(2-dimethylaminoethoxy)ethane	2.83	1.2	–	–	–	–	7	8
Bis(2-dimethylaminoethyl) ether	1.23 (0.94 - 1.62)	0.28	8 hr.	–	–	–	6	9
Cyclohexylamine	0.71 (0.51 - 0.97)	0.32 (0.24 - 0.43)	2 hr.	4,000†	4	0/6	7	10
1,8-Diamino-p-menthane	0.77 (0.62 - 0.95)	0.63 (0.47 - 0.85)	4 hr.	–	–	–	7	9
2,4-Diamino-1-methylcyclohexane	1.41	0.50 (0.31 - 0.82)	8 hr.	–	–	–	6	9
Di(3-aminopropyl) ether of diethylene glycol	4.29 (3.07 - 5.98)	2.50 (1.14 - 5.48)	4 hr.	–	–	–	6	8
Di(3-aminopropoxy)ethane	2.83 (1.76 - 4.55)	1.25 (0.84 - 1.85)	8 hr.	–	–	–	6	8
N,N-Di(methyl-N-cyclohexylmethyl)amine	1.23 (0.94 - 1.62)*	0.21 (0.15 - 0.30)	8 hr.	–	–	–	7	9
N-(Methyl-N-cyclohexylmethyl)amine	1.41 *	0.63 (0.47 - 0.85)	4 hr.	–	–	–	6	9
N,N,N',N',N''-Pentamethyldiethylenetriamine	1.63 (1.24 - 2.13)	0.28	8 hr.	–	–	–	7	9
N,N,N',N'-Tetramethyl-1,3-butanediamine	0.93 (0.67 - 1.30)	0.40 (0.29 - 0.54)	8 hr.	–	–	–	6	9
N,N,N',N'-Tetramethyl-1,3-propanediamine	0.41 (0.31 - 0.53)	0.30 (0.21 - 0.42)	15 m.	1,000	4	2/6	5	9
Tetramethyl-2-tetrazene	1.78 (1.12 - 2.83)	—	4 hr.	–	–	–	2	4
Tripropylamine	0.096(0.071- 0.13)*	0.57 (0.26 - 1.23)	8 hr.	250	4	3/6	4	1
Aromatic Nitro and Amino Compounds								
3-Aminopyrene	1.07 (0.77 - 1.50)*	> 1	8 hr.	–	–	–	–	8
N-Methyl-1-naphthylamine	1.41 (1.03 - 1.95)		8 hr.	–	–	–	2	1

TABLE I
Range-Finding Toxicity Data (Continued)

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corneal Injury in Rabbits
Amides								
N(2-Acetoxyethyl)-N-ethylacetamide	20.8 (16.8 -25.7)	>10	8 hr.	–	–	–	2	4
e-Caprolactam	2.14 (1.54 - 2.99)*	1.41	4 hr.	–	–	–	–	7
N-Ethyl-N-vinylacetamide	2.46 (1.88 - 3.23)	1.25 (0.84 - 1.85)	4 hr.	2,000	4	3/6	2	3
Propyl-N,N-diethylsuccinamate	12.93 (8.42 -19.87)	>20	8 hr.	–	–	–	4	5
Heterocyclic Compounds								
3,5-Dimethyl-1-(trichloromethylmercapto)-pyrazole	0.57 *	0.20 (0.15 - 0.27)*	4 hr.	–	–	–	6	10
Isopropylmorpholine	0.71	0.10 (0.07 - 0.15)	15 m.	500	4	5/6	6	7
Isopropylpiperazine	2.83	0.64 (0.47 - 0.86)	8 hr.	–	–	–	6	9
3,4-Lutidine	0.71 (0.40 - 1.24)	0.14	1 hr.	250†	4	0/6	6	8
N-Methyl-e-caprolactam	1.62 (1.18 - 2.24)	1.41	8 hr.	–	–	–	3	5
3-Methyl-2-oxazolidone	7.13 (3.32 -15.30)	10.8 (6.17 -16.5)	8 hr.	–	–	–	1	4
2-Methyl-5-vinylpyridine	1.30 (0.71 - 2.37)	0.80 (0.54 - 1.18)	4 hr.	4,000	8	4/6	5	8
1-(Trichloromethyl mercapto) pyrazole	0.35 *	0.063(0.047- 0.085)	–	200	1	6/6	7	9
1-(Trichloromethyl-mercapto)-4-methylpyrazole	1.04 (0.81 - 1.28)	0.079(0.059- 0.107)	2 m. §	–	–	–	7	8
Cyano Compounds								
Bis(2-isocyanatoethyl)carbonate	1.83 (1.21 - 2.79)	>12	8 hr.	–	–	–	3	9
Bis(2-isocyanatoethyl)-4-cyclohexene-1,2-dicarboxylate	10.4 (5.55 -19.4)	> 8	8 hr.	–	–	–	5	5
Bis(2-isocyanatoethyl)-5-norbornene-2,3-dicarboxylate	22.6 (13.4 -38.2)	16.1 (4.5 -57.2)	8 hr.	–	–	–	5	5
3-Butenenitrile	0.115(0.097- 0.138)*	1.41	5 m.	250†	4	0/6	2	3
N,N-Dibutyl-3-aminopropionitrile	3.25 (2.48 - 4.26)	5.04 (3.73 - 6.82)	8 hr.	–	–	–	2	2
1,6-Hexamethylene diisocyanate	0.71	0.57 (0.35 - 0.91)	8 hr.	–	–	–	8	9
Lactonitrile	0.087(0.060- 0.125)*	0.020(0.015- 0.027)	–	62.5†	4	0/6	2	–‡
3-Methoxypropionitrile	4.39 (3.07 - 5.98)*	>10	8 hr.	–	–	–	2	5
Methyl isocyanate	0.071(0.027- 0.18)*	0.22 (0.12 - 0.41)	–	31.25†	4	0/6	6	10
Tridecanitrile, mixed isomers	3.73 (2.68 - 5.21)	3.18 (1.43 - 7.06)	8 hr.	–	–	–	4	5
Halogen Compounds								
1-Bromooctane	4.49 (2.09 - 9.64)	8.00 (4.93 -13.0)	–	–	–	–	5	1
2-Chlorobutane	20.0 (12.8 -31.3)	20	5 m. §	8,000	4	3/6	1	2
Chloromethyl ether	0.21 (0.12 - 0.37)	0.28 (0.13 - 0.62)	–	8†	4	1/6	6	10
Dibromobicycloheptane, mixed isomers	0.21 (0.15 - 0.30)*	0.25 (0.19 - 0.34)	1 hr.	–	–	–	6	–
Dichlorobenzyl alcohol, mixed isomers	0.81 (0.50 - 1.31)	0.40 (0.24 - 0.65)	8 hr.	–	–	–	5	9
1,2-Dichloropropane(propylene dichloride)	1.9 (1.7 - 2.1)	8.75 (8.31 - 9.20)	10 m.	2,000	8	3/6	1	2
trans-2,3-Dichloro-1,4-dioxane	1.41	0.44 (0.27 - 0.71)	4 hr.	–	–	–	5	7
1,2-Dichloroethane(ethylene dichloride)	0.77 (0.67 - 0.89)*	3.89 (3.40 - 4.46)	–	–	–	–	2	3
Di(2-chloroethyl)acetal	0.31 (0.24 - 0.40)*	0.20 (0.14 - 0.30)	1 hr.	–	–	–	3	2
Fumaroyl chloride	0.81 (0.51 - 1.30)	1.41	–	500	4	3/6	6	10
1,1,1,3,3,3-Hexachloro-2,2-difluoropropane	0.54 (0.30 - 1.01)	4.53 (2.80 - 7.32)	–	–	–	–	3	2
m-Nitrobenzoyl chloride	2.46 (1.79 - 3.39)	0.79 (0.49 - 1.20)	[illegible]	–	–	–	5	9

(Continued on Next Page)

TABLE I
Range-Finding Toxicity Data (Continued)

1-Bromooctane	4.49 (2.09 - 9.64)	[illegible]	[illegible]	–	–	–	[illegible]	[illegible]
2-Chlorobutane	20.0 (12.8 -31.3)	29	5 m. §	8,000	4	3/6	[illegible]	[illegible]
Chloromethyl ether	0.21 (0.12 - 0.37)	0.28 ([illegible] - [illegible])	–	8†	4	1/6	6	10
Dibromobicycloheptane, mixed isomers	0.21 (0.15 - 0.30)*	0.25 (0.19 - 0.34)	1 hr.	–	–	–	6	–
Dichlorobenzyl alcohol, mixed isomers	0.81 (0.50 - 1.31)	0.40 (0.24 - 0.65)	8 hr.	–	–	–	5	9
1,2-Dichloropropane (propylene dichloride)	1.9 (1.7 - 2.1)	8.75 (8.31 - 9.20)	10 m.	2,000	8	3/6	1	2
trans-2,3-Dichloro-1,4-dioxane	1.41	0.44 (0.27 - 0.71)	4 hr.	–	–	–	5	7
1,2-Dichloroethane (ethylene dichloride)	0.77 (0.67 - 0.89)*	3.89 (3.40 - 4.46)	–	–	–	–	2	3
Di(2-chloroethyl)acetal	0.31 (0.24 - 0.40)*	0.20 (0.14 - 0.30)	1 hr.	–	–	–	3	2
Fumaroyl chloride	0.81 (0.51 - 1.30)	1.41	–	500	4	3/6	6	10
1,1,1,3,3,3-Hexachloro-2,2-difluoropropane	0.54 (0.30 - 1.01)	4.53 (2.80 - 7.32)	–	–	–	–	3	2
m-Nitrobenzoyl chloride	2.46 (1.79 - 3.39)	0.79 (0.49 - 1.30)	8 hr.	–	–	–	5	9

(Continued on Next Page)

TABLE I

Range-Finding Toxicity Data (Continued)

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corneal Injury in Rabbits
Phenyl chloroformate	1.41 (0.83 - 2.40)	3.97 (2.93 - 5.37)	15 m.	44	4	3/6	6	10
Propylene chlorohydrin	0.22 (0.19 - 0.27)	0.48 (0.34 - 0.67)	15 m.	500	4	1/6	1	8
Tetrachloroethane	0.20 (0.16 - 0.27)	3.99 (3.10 - 5.13)	–	1,000	4	3/6	6	–
1,1,2,3-Tetrachloropropene	0.35 *	0.40 (0.27 - 0.59)	30 m.	250	4	5/6	5	9
1,1,2-Trichloroethane	0.58 (0.47 - 0.71)	3.73 (3.30 - 4.21)	–	500	8	4/6	2	2
Trichloroethylene	4.92 (3.75 - 6.46)	>20	–	8,000	4	4/6	5	4
Phosphorous Compounds								
Bis(2-chloroethyl)phosphite	0.26 (0.19 - 0.35)*	0.141(0.065- 0.308)	8 hr.	–	–	–	2	3
Diethyl phosphite	5.19 (2.81 - 9.58)	2.02 (1.24 - 3.29)	4 hr.	–	–	–	1	5
Trimethyl phosphate	2.83 (1.75 - 4.57)	2.83	8 hr.	–	–	–	4	2
Tris(dipropylene glycol)phosphine	19.7 (15.0 -25.8)	>10	8 hr.	–	–	–	2	1
Tris(dipropylene glycol)phosphonate	29.9 (21.4 -41.6)	15.9 (11.7 -21.5)	8 hr.	–	–	–	3	1
Sulphur Compounds								
Bis(2-acetoxyethyl)sulfone	14.1	11.3	8 hr.	–	–	–	4	2
Bis(1,2-dichloroethyl)sulfone	0.25 (0.19 - 0.32)*	1.00 (0.40 - 2.51)	8 hr.	–	–	–	7	9
2,3-Epithiopropyl methoxy ether	2.14 (1.54 - 2.99)	> 5	1 hr.	–	–	–	3	2
n-Hexyl vinyl sulfone	0.57	0.84 (0.50 - 1.40)	–	–	–	–	7	5
Isopropyl sulfate	1.09 (0.64 - 1.87)	1.41 (0.88 - 2.29)	1 hr.	–	–	–	6	5
2(Methoxyethylthio)ethanol	13.00 (7.70 -21.93)	5.04 (3.09 - 8.23)	8 hr.	–	–	–	2	5
Methylvinyloxyethyl sulfide	3.25 (2.48 - 4.26)	> 8	–	3,200	8	4/6	2	2
Tetrahydrothiophene-1,1-dioxide (sulfolane)	1.54 (1.25 - 1.91)	3.18 (2.35 - 4.29)	8 hr.	–	–	–	2	4
2,2'-Thiodiethanol diacetate	9.25 (7.51 -12.9)	>16	8 hr.	–	–	–	3	1
Trichloromethyl allyl perthioxanthate	2.18 (1.39 - 3.42)*	0.6	–	–	–	–	6	1
Trichloromethyl methyl perthioxanthate	1.54 (0.89 - 2.68)*	1.3	–	–	–	–	6	2
Silicon Compounds								
Amyl trimethoxy silane	4.92 (3.75 - 6.46)	10.00 (4.56 -21.9)	4 hr.	–	–	–	4	1
2(3,4-Epoxycyclohexyl)ethyl trimethoxy silane	12.3 (9.98 -15.3)	6.30 (4.66 - 8.52)	8 hr.	–	–	–	3	1
3(2,3-Epoxypropoxy)propyl trimethoxy silane	22.6	3.97 (2.93 - 5.37)	8 hr.	–	–	–	3	2
2-Ethylbutyl silicate	19.7 (15.0 -25.8)	>10	8 hr.	–	–	–	3	1
2-Mercaptoethyl trimethoxy silane	2.46 (1.88 - 3.23)	–	2 hr.	–	–	–	3	2
3-Mercaptopropyl trimethoxy silane	2.83 (1.61 - 4.98)	5.66	–	–	–	–	3	2
Methyl trimethoxy silane	12.5 (9.98 -15.3)	>10	1 hr.	–	–	–	3	2
Trimethoxy silane	9.33 (6.69 -13.0)	6.30 (4.66 - 8.52)	15 m.	62.5	4	5/6	3	5
Vinyl trimethoxy silane	11.3	3.54 (2.03 - 6.16)	2 hr.	4,000	4	1/6	2	1

* as gm/kg in a suitable vehicle

§ inhalation time shown killed all six rats

† twice concentration shown killed all six rats

‡ caused death when introduced into the rabbit eye

TABLE II
Acute Oral Toxicity of Some Inorganic Compounds for Rats

Material*		Formula	Single Oral LD_{50} for Rats gm/Kg	Concentration Intubated gm/ml**	
Aluminum isopropylate	T	$Al[OCH(CH_3)CH_3]_3$	11.3 (7.0 -18.3)	0.200	A
Aluminum nitrate		$Al(NO_3)_3 . 9H_2O$	4.28 (3.86 - 4.76)	0.100	
Ammonium metavanadate	T	NH_4VO_3	0.16 (0.12 - 0.21)	0.0025	B
Ammonium paratungstate	T	$(NH_4O)_6W_7O_{18} . 6H_2O$	11.3	0.400	A
Ammonium persulfate		$(NH_4)_2S_2O_8$	0.82 (0.53 - 1.26)	0.200	
Borax		$Na_2B_4O_7 . 10H_2O$	5.66	0.010	
Boric acid		H_3BO_3	5.14 (4.75 - 5.58)	0.200	
Calcium acetate		$Ca(OOCCH_3)_2 . H_2O$	4.28 (3.86 - 4.76)	0.100	
Calcium acrylate	T	$Ca(OOCCH{=}CH_2)_2 . H_2O$	4.92 (3.75 - 6.46)	0.200	A
Calcium cyanide		$Ca(CN)_2$	0.039(0.030- 0.051)	0.001	
Calcium hydroxide		$Ca(OH)_2$	7.34 (4.83 -11.14)	0.100	
Calcium propionate	T	$Ca(OOCCH_2CH_3)_2 . H_2O$	5.16 (4.17 - 6.38)	0.100	
Chromium acetate	T	$Cr(OOCCH_3)_2 . H_2O$	11.26	0.200	
Chromium chloride (ous)	T	$CrCl_2$	1.87 (1.21 - 2.87)	0.050	
Chromium nitrate (ic)		$Cr(NO_3)_3 . 9H_2O$	3.25 (2.11 - 4.99)	0.200	
Cobalt oxide	T	CoO	1.70 (1.07 - 2.82)	0.100	A
Copper acetate		$Cu(OOCCH_3)_2 . H_2O$	0.71 (0.60 - 0.83)	0.010	
Copper nitrate		$Cu(NO_3)_2 . 3H_2O$	0.94 (0.61 - 1.43)	0.200	
Copper sulfate		$CuSO_4 . 5H_2O$	0.96 (0.71 - 1.30)	0.050	
Cuprous oxide	T	Cu_2O	0.47 (0.34 - 0.66)	0.100	A
Diammonium decaborane	T	$(NH_4)_2B_{10}H_{10}$	3.56 (1.85 - 6.86)	0.050	
Dicumene chromium	T	$(C_9H_{12})_2Cr$	0.81 (0.62 - 1.07)	0.100	M
Ferric nitrate		$Fe(NO_3)_3 . 9H_2O$	3.25 (2.56 - 4.26)	0.200	
Ferrous ammonium sulfate		$(NH_4)_2SO_4 . FeSO_4 . 6H_2O$	3.25 (2.48 - 4.26)	0.300	
Lithium carbonate		Li_2CO_3	0.71	0.200	
Magnesium chloride		$MgCl_2 . 6H_2O$	8.1 (7.3 - 9.1)	0.100	
Manganous, acetate		$Mn(OOCCH_3)_2 . 4H_2O$	3.73 (2.68 - 5.21)	0.200	
Nickel nitrate		$Ni(NO_3)_2 . 6H_2O$	1.62 (1.06 - 2.50)	0.200	
Potassium acetate		$KOOCCH_3$	3.25 (2.48 - 4.26)	0.100	
Potassium carbonate		K_2CO_3	1.87 (1.34 - 2.60)	0.200	
Potassium hydroxide		KOH	1.23 (0.80 - 1.89)	0.100	
Potassium permanganate		$KMnO_4$	1.09 (0.70 - 1.71)	0.020	
Silver oxide	T	Ag_2O	2.82	0.200	A
Sodium arsenite		$NaAsO_2$	0.041(0.031- 0.053)	0.010	
Sodium cyanide		$NaCN$	0.015(0.011- 0.021)	0.0004	
Sodium fluoride		NaF	0.18 (0.12 - 0.26)	0.005	
Sodium nitrite		$NaNO_2$	0.18	0.010	
Sodium phosphate, diabasic		$Na_2HPO_4 . 7H_2O$	12.93 (9.85 -16.97)	0.200	
Sodium phosphate, tribasic		$Na_3PO_4 . 12H_2O$	7.40 (6.81 - 8.03)	0.200	
Sodium tripolyphosphate	T	$Na_5P_3O_{10}$	6.50 (4.03 -10.48)	0.200	
Sulfuric acid		H_2SO_4	2.14 (1.54 - 2.99)	0.250	
Vanadium dichloride	T	VCl_2	0.54 (0.38 - 0.75)	0.010	A
Vanadium oxytrichloride	T	$VOCl_3$	0.14 (0.09 - 0.22)	0.050	M
Vanadium tetrachloride	T	VCl_4	0.16	0.090	M
Vanadium trichloride	T	VCl_3	0.35 (0.22 - 0.57)	0.100	
Zinc acetate		$Zn(OOCCH_3)_2 . 2H_2O$	2.46 (1.60 - 3.78)	0.200	
Zinc naphthenate	T		4.92 (3.75 - 6.46)	0.200	L

* T Preceding formula designates technical grade, or development sample, otherwise reagent grade

** Intubation concentrations are in water except as noted by following symbols:

M In mineral oil

L In lard

A Suspended in 0.25% agar

B Suspended in 5% bentonite

Transfer 2 ml each soln to each of 3 test tubes. Designate as std or sample tubes.

At appropriate time, dil. blank solns to vol. with H_2O. Transfer 2 ml of these solns to each of 3 test tubes. Designate as std or sample blank tubes.

Using automatic pipetting machine, add 5 ml *o*-phenylenediamine soln to all tubes. Use Vortex mixer to swirl tubes. Protect from light and let stand 35 min. at room temp. Transfer solns to fluorescence reading tubes.

Fluorometry

Measure fluorescence of std tube (*A*), std blank tube (*B*), sample tube (*C*), and sample blank tube (*D*).

mg Ascorbic acid/g, tablet, ml, etc. = [(av. C − av. D)/(av. A − av. B)] × [V/(10 × E)], where V = initial assay soln vol. and E = no. of g, tablets, ml, etc. assayed.

40. RADIOACTIVITY

No additions, deletions, or other changes.

41. SPECTROSCOPIC METHODS

No additions, deletions, or other changes.

42. STANDARD SOLUTIONS

No additions, deletions, or other changes.

ERRATA AND EMENDATIONS, OFFICIAL METHODS OF ANALYSIS, AOAC, 1965

The following changes reported by users of this book should be made:

Section	*Page*	
4.048	43	Line 15, after "Ignite" insert "at 950°"
4.131	53	Last sentence, change to read "Ignite at 950° in Pt crucible to constant wt (CaO) and calc. % Ca."
4.195	65	Line 2, change "3.455" to "3.545"
7.023	122	Line 5, after "lamp" insert "at not <950°"
14.003	215	Last sentence, change to read "Proceed as in 14.020 or 14.021"
16.041	264	Line 1, change "Place 5 g H_2SiO_3 in" to "Place 5 g silicic acid, 18.017(g), in"
16.046	266	Lines 3 and 4, change "centrifuge" to "swirl"
20.031(a)	313	Line 1, change "MoO" to "MoO_3"
31.024	525	Line 13, after "lamp" insert "at not <950°"
		Line 21, after "ignite" insert "at 950°"
31.061	532	Line 17, after "lamp" insert "at not <950°"
32.077	549	After "in 32.015" insert "except use 1*N* HCl in place of 1*N* HNO_3 in prepg Column II."
33.005	632	Lines 12–14, change "Read absorbance against H_2O at wavelength of max. absorption available in spectrophotometer used (750 mμ or above)" to "Read absorbance against H_2O at 750 mμ or above (max. absorption is at 810 mμ)"
33.131(a)	656	Line 2, after "chlortetracycline.HCl NF" insert "Reference Standard"; delete "of known potency"
33.139	657	Change to read "Units/lb = [1.1 × (units/ml assay soln) × 454 × ml pH 7 buffer (300 or 500) × 10 × (125 + ml HCl + ml 40% NaOH)]/[125 ml × g sample × ml neutralized soln put on column]"
33.148(b)	659	Line 4, after "N.Y. 11206)" insert "or NF Reference Standard"
33.152(a)	660	Line 2, after "oxytetracycline NF" insert "Reference Standard"

The following corrections should be made in *Changes in Methods, This Journal* 49, February 1966:

Page	
209	Right column, line 5 of text, after "KOH absorber" insert "and bubblers"
220	Left column, 19. *Flavors*, Item (1), line 4 of revised 19.088, change "Pipet 3 ml into 100 ml vol. flask." to "Pipet 3 ml into 1 L vol. flask,"; line 9, change equation to read "$A_{318} - (0.29\ A_{270} + 0.71\ A_{350})$"
	Right column, line 2 of text, change "0.1 ml 1*N* KOH," to "0.1 ml KOH soln

OILS, FATS, AND WAXES

The Multi-Determination of Antioxidants in Lard

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A method has been developed for the multiple determination of antioxidants (BHA, BHT, Ionox-100, PG, THBP, and TDPA) in lard. The procedure consists of vacuum sublimation of the antioxidants and quantitative determination by gas chromatography. UV, IR, and mass spectrometry were used for identification and confirmation. Average recoveries of the antioxidants added to 4 g lard at 100 ppm ranged from 91 to 96%.

Under present food additive regulations, various antioxidants are acceptable for addition, either directly to food products or indirectly through the medium of food packaging materials. These antioxidants are 3-(2-)*tert.*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert.*-butyl-4-hydroxytoluene (BHT), *n*-propyl gallate (PG), nordihydroguaiaretic acid (NDGA), 2,4,5-trihydroxybutyrophenone (THBP), 3-3′-thiodipropionic acid (TDPA), dilaurylthiodipropionate (DLTDP), and gum guaiac. More recently 4-hydroxymethyl-2,6-di-*tert.*-butylphenol (Ionox-100) has been proposed for use.

In recent years, mixtures of antioxidants have been used extensively, since no single compound is equally effective in all substrates under all conditions, and best results are usually obtained with combinations. It is important, therefore, to have a rapid analytical method which can determine these compounds in food products.

At the present time the most widely used analytical procedures for the determination of antioxidants are colorimetric methods (1–8). Many of these procedures are subject to limitations of nonspecificity, varying degrees of sensitivity and precision, and interference by other antioxidants. In 1956, Anglin, *et al.* (1) described a colorimetric method for the quantitative estimation of BHA, BHT, PG, and NDGA in edible fats. In this method, PG and NDGA are extracted with 72% ethyl alcohol from fat dissolved in petroleum ether or carbon tetrachloride, whereas BHT and BHA are steam distilled. The antioxidants PG and NDGA, however, cannot be estimated in mixtures. Excellent recoveries were obtained of antioxidants added to lard at levels as low as 25 ppm for NDGA and 50 ppm for BHA, BHT, and PG.

In 1964, Sahasrabudhe (9) reported on the quantitative estimation of BHA, BHT, PG, and NDGA in lard. The antioxidants were extracted from *n*-hexane solution of the fat with 80% ethyl alcohol and acetonitrile and separated by two-dimensional thin layer chromatographic techniques. Quantitative measurements were made by colorimetric methods after extraction of the compounds from the adsorbent. Recoveries of antioxidants added to lard at levels of 50 to 200 ppm ranged from 94 to 102%. The advantages of this method, as cited by the author, are that (*1*) all four antioxidants can be estimated in one aliquot of fat, (*2*) PG and NDGA can be estimated when present together in mixtures, and (*3*) the isomers of BHA can be effectively separated.

Gas chromatographic procedures have also been used to determine BHA and BHT in food products. Buttery and Stuckey (10), in 1961, employed a direct extraction of these antioxidants from potato granules fol-

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lowed by gas chromatography of the eluate on an Apiezon L column with flame ionization detection. A range of 0.5 to 10 ppm of antioxidant was analyzed with an average error of less than 11%. Schweeke and Nelson (11) and Anderson, *et al.* (12) have also determined BHA and BHT in cereals, potato products, and waxed paper by gas chromatography. These workers used a silicone gum (2% SE-30/1% Tween 80) column and a flame ionization detector. Recoveries of levels in the 5–30 ppm range varied from 95 to 103%.

In a recent publication, Schwien, *et al.* (13) described an acetonitrile extraction and a subsequent cleanup on an alumina acid column for the separation of BHA and BHT from fats and oils prior to determination by gas chromatography. Recoveries from tallow, lard, and corn oil spiked at 16.7 and 66.7 ppm with BHA and BHT ranged from 80 to 95% for BHA and 91 to 115% for BHT.

This report describes a quantitative procedure for the determination of BHA, BHT, PG, Ionox-100, THBP, and TDPA in lard. Gum guaiac was omitted from this study since available information indicates that it is no longer used in the United States. In subsequent investigations, this method will be extended to NDGA and DLTDP and to a wide range of food products.

METHOD

Apparatus

(a) *Vacuum and diffusion pumps.*—(*1*) Welch Duo-Seal (Model 1402B, Welch Scientific Co., Skokie, Ill.), or equivalent, and (*2*) Veeco diffusion pump (Model EP-2-5W, Veeco Instrument Co., Inc., Plainview, N.Y.), or equivalent.

(b) *Recording spectrophotometer and accessories.*—(*1*) Cary 11 (Applied Physics Corp., Monrovia, Calif.), or equivalent; (*2*) fused quartz cells, 1 cm optical path length and 0.35 ml capacity (Optical Cell Co., Inc., Brentwood, Md.), or equivalent.

(c) *Infrared spectrophotometer and accessories.*—Beckman IR-5A, with 5x beam condenser and NaCl ultra-micro liquid cells, 0.1 mm path length, (24196, Beckman Instruments Co., Fullerton, Calif.), or equivalent.

(Custom built by Laboratory Supply and Equipment Co., Chevy Chase, Md.): (*1*) Flat bottom distillation flask: fabricated from a 25 mm O-ring plain joint #6780, Corning Glass Works, Corning, N.Y.). The plain end is sealed off and flask is custom blown to shape. The flask is 52 mm high and has an o.d. of 52 mm at the widest point. (*2*) Column: 102 mm long, fabricated by fusing the plain ends of two 25 mm O-ring joints (#6780, Corning Glass Works) together. Baffles are formed into the lower 45 mm portion of the column in 2 tiers of three, equidistant from each other. Each baffle is produced by pushing the molten glass wall of the column inward with a forming tool. (*3*) Cold finger section: made by fusing the plain end of a 9 mm O-ring joint (#6780, Corning Glass Works) perpendicular to the plain end of a 25 mm O-ring joint (#6780, Corning Glass Works). A 110 mm length of 16 mm o.d. glass tubing is centered inside the 25 mm joint and fused onto the plain end. The finger is formed by sealing off the tube and shaping. Dimensions of unit are as follows: overall length of the cold finger = 110 mm, length from top of unit to joint lower edge = 38 mm, and length of side arm joint = 30 mm. (*4*) Insulated bath: consists of two Pyrex glass beakers placed one inside the other. The inner beaker (800 ml capacity tall form) containing ca 500 g silicone fluid (or other heat transfer liquid) is wrapped in heating tape and is embedded in sand contained by the second beaker (2 L capacity Griffin low form). Fiber glass insulation material (1" thick) is wrapped around the 2 L beaker to prevent heat loss. (*5*) Clamps: custom built, C-shaped, held together by brass screws.

(e) *Stirrer-hot plate.*—Model 11-493 V2 (Fisher Scientific Co., Silver Spring, Md.), or equivalent.

(f) *Volumetric flask.*—1 ml capacity (K-62150, Kontes Glass Co., Vineland, N.J.), or equivalent.

(g) *Concentrator tube.*—4 ml capacity (K-57005, Kontes Glass Co.), or equivalent.

(h) *GLC collection trap.*—10 ml capacity, custom built (Laboratory Supply and Equipment Co.). *See* Fig. 2 for detailed drawing: the inside diameter of the inner tube is just large enough to admit the barrel of a 10 µl Hamilton syringe.

(i) *Syringes.*—10, 25, and 100 µl (Hamilton Co., Inc., Whittier, Calif.), or equivalent.

(j) *Ion gauge.*—Vacronic Laboratory Equipment, Inc., East Northport, N.Y., or equiva-

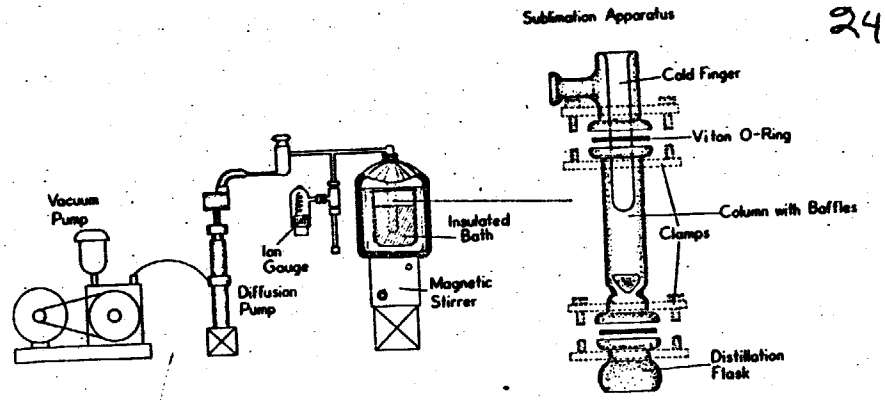

Fig. 1—Sublimation apparatus.

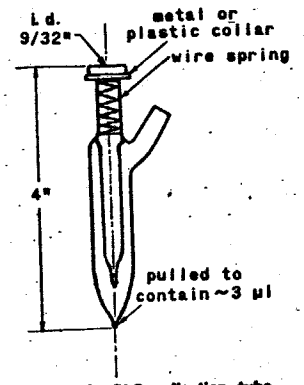

Fig. 2—GLC collection tube.

(k) *Gas chromatograph.* — Barber-Colman Model 5000 with a flame detector, fraction collector, and effluent splitter (20:1 ratio) (Barber-Colman Co., Rockford, Ill.).

Column and conditions: Pack 16' × 3 mm column with 3% GE-XE-60 liquid phase (nitrile-silicone gum rubber, Analabs, Hamden, Conn.) on 60/80 mesh Gas Chrom-Q (Applied Science Laboratories, Inc., State College, Pa.); precondition by heating the column for 48 hr at 250°C while maintaining a carrier gas (nitrogen) flow of 30 ml/min. Maintain the following parameters throughout the analyses: injector temperature, 130–250°C; detector temperature, 260°C; carrier gas flow rate, 125 ml/min.; hydrogen flow rate, 37 ml/min., air or oxygen flow rate, 500 ml/min.; and attenuation range, X2 at 3 × 10^{-9} amp. into 1 mv full scale recorder.

The three column temperature programs are *Program I*, from 100 to 150°C at 10°/min. and then to 250°C at 5°/min.; *Program II*, from 100 to 165°C at 10°/min., then operate at 165°C isothermally for 16 min. followed by a manual rise to 250°C and again run isothermally for 10 min.; and *Program III*, from 90 to 130°C at 10°/min., then operate at 130°C isothermally for 10 min.

Reagents

(a) *Ethyl ether.*—Analytical reagent (Mallinckrodt Chemical Works, St. Louis, Mo.), or equivalent.

(b) *Pyridine.* — ACS reagent (Baker and Adamson, New York, N.Y.), or equivalent.

(c) *Methanol.*—Reagent, anhydrous (Merck & Co., Rahway, N.J.), or equivalent.

(d) *Antioxidants.*—Tennox PG (propyl gallate), Tennox BHA (butylated hydroxyanisole), and Tennox BHT (butylated hydroxytoluene), food grade (Eastman Chemical Products, Inc., Kingsport, Tenn.), or equivalent; 2,4,5-trihydroxybutyrophenone (THBP), experimental grade (Eastman Chemical Products, Inc.), or equivalent; Ionox-100 (4-hydroxy methyl-2,6-di-*tert.*-butyl phenol), food grade (Shell Chemical Co., Division of Shell Oil Co., Union, N.J.), or equivalent; thiodipropionic acid (TDPA) (Carlisle Chemical Works, Reading, Ohio), or equivalent.

(e) *Standard solutions of antioxidants.* — Transfer 1 g of each antioxidant (PG, BHA, BHT, THBP, and Ionox-100) to individual 100 ml volumetric flasks and dilute to volume with

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anhydrous ethyl ether. Transfer 2 ml of each stock solution to separate 100 ml volumetric flasks and adjust to volume with anhydrous ethyl ether. (Each solution contains 0.2 mg antioxidant/1 ml.)

Dissolve 1 g TDPA in 25 ml methanol in a 100 ml volumetric flask and dilute to volume with anhydrous ethyl ether. Transfer 2 ml of this stock solution to a 100 ml volumetric flask and adjust to volume with anhydrous ethyl ether. (This solution contains 0.2 mg TDPA/1 ml.)

(f) *Chlorotrimethylsilane.*—Eastman Chemical Products, Inc., Kingsport, Tenn., or equivalent.

(g) *1,1,1,3,3,3-Hexamethyldisilazane.* — Eastman Chemical Products, Inc., Kingsport, Tenn., or equivalent.

(h) *Diazald (N-methyl-N-nitroso-p-toluene sulfonamide).*—Aldrich Chemicals Co., Milwaukee, Wis., or equivalent. Use to prepare diazomethane, according to manufacturer's directions, as follows:

Prepare reagent in a well-ventilated hood. Use cork stoppers (14) for all connections. Fit a 125 ml long-neck distilling flask with a dropping funnel and an efficient condenser. Connect condenser in series to a 500 ml and a 125 ml Erlenmeyer receiving flask, respectively. Place 20 ml ether in the 125 ml receiver and adjust height of inlet tube to below the surface of the ether. Cool both receivers to 0°C.

Dissolve 6 g KOH in 10 ml water in the distilling flask. Add 35 ml 2-(2-ethoxyethoxy)-ethanol (Aldrich) and 10 ml ether. Connect distilling flask to condenser and immerse flask in a water bath maintained at 70°C with a magnetic stirrer-hot plate. Start stirrer and, as distillation begins, add a solution of 21.5 g "Diazald" in 140 ml ether through the dropping funnel over 20-min. period. At end of distillation, add another 20 ml ether and continue to distill until distillate is colorless. Combine contents of the two receivers. Freeze overnight. Decant the diazomethane from the ice crystals into a polyethylene or glass bottle. Stopper with a cork. Store in freezer until ready for use. (*Caution:* Diazomethane is both toxic and explosive.)

(i) *Silicone fluid.*—F-1-0173, 100 cst (Dow-Corning Corp., Midland, Mich., or equivalent).

Procedures

Vacuum separation.—Weigh 4 g lard sample into a 60 ml flat-bottom distillation flask. Attach flask to a glass column containing the built-in baffle system and a cold finger, and connect the sublimation apparatus to the vacuum system as shown in Fig. 1. Fill the cold finger with liquid nitrogen. Maintain liquid N level during sublimation. Start vacuum pump and slowly apply vacuum to the system. (*Note:* The pressure in the system should drop to about 0.5 μ as measured by the gauge attached between the cold finger and the trap.) Lower assembly into insulated bath maintained at 188°C. After 2 hr discontinue vacuum and remove assembly from the silicone oil bath.

Remove retaining clamps and cautiously lift out the cold finger. (*Caution:* Losses occur by accidental scraping of cold finger against the walls of the column.) Pour off liquid nitrogen. Quantitatively transfer the sublimate to the concentrator tube by washing the cold finger with small portions of anhydrous ethyl ether, using a 1 ml hypodermic syringe. Adjust volume to 2 ml by evaporating excess solvent under nitrogen in a warm water bath. Mix and reltain for gas-liquid chromatographic analysis. The entire solution will be required for the quantitative determination of BHA, BHT, Ionox-100, PG, THBP, and TDPA.

Gas liquid chromatography.—Inject 20 μl ether solution of the sublimate to be analyzed into the column, program the temperature according to *Program I*, and collect the effluents corresponding to BHA, BHT, or Ionox-100 in the 10 ml collection trap cooled with liquid nitrogen. Retain for identification by ultraviolet spectral analysis.

For the determination of PG and THBP, pipet 1 ml ether solution of the sublimate in the concentrator tube into a 1 ml volumetric flask. Evaporate solvent almost to dryness in a warm water bath under nitrogen. Adjust volume to 1 ml with the silanizing reagents as follows: 0.5 ml pyridine, 0.3 ml 1,1,1,3,3,3-hexamethyldisilazane, and 0.2 ml chlorotrimethylsilane. Stopper flask, mix well, and let solution stand for 5 min. Inject 20 μl of solution into the column, program according to *Program II*, and collect the PG and THBP effluents in separate 10 ml traps cooled with liquid nitrogen. Retain for confirmation of identity by ultraviolet spectral analysis.

To determine TDPA, use the remaining ether solution in the concentrator tube. Evaporate the solvent to a small volume (ca 0.3 ml) in a warm water bath under nitrogen. Adjust to 1 ml with diazomethane and mix. Inject 30 μl of solution into the chromatograph and program the temperature according to *Program III*. If a peak is obtained (indicating the presence of TDPA), inject a 250 μl aliquot and collect the effluent in the 10 ml liquid nitrogen-cooled fraction collector. Retain for identification by infrared spectrophotometry.

For the quantitation of the antioxidants prepare the standardization curves as follows:

For BHA, BHT, and Ionox-100, inject appropriate aliquots of the standard solutions directly into the gas chromatograph. Use 1 ml aliquots of PG, THBP, and TDPA standard solutions, evaporate each solution under nitrogen, and prepare the silyl and methoxylated derivatives as described above. Collect the effluent corresponding to each antioxidant under study and reserve for confirmatory analysis. Calculate the amounts of the antioxidants present by either the peak height or peak area techniques.

To confirm the identity of BHA, BHT, Ionox-100, PG, and THBP, add 0.4 ml methanol to each of the traps containing the column effluents and swirl. Transfer solutions to 0.35 ml capacity 1 cm path length cells and record the ultraviolet spectra in the wavelength region of 210–400 mμ, using methanol in the reference cell. Compare with spectra obtained for the standards under identical instrumental conditions.

To confirm the identity of TDPA, wash the trap with CS_2 and evaporate the solvent to about 3 μl. Transfer the solution to a Beckman 0.1 ml micro liquid cell and record the infrared spectrum, using the Beckman IR 5A spectrophotometer equipped with a 5x beam condenser. Compare with the spectrum obtained for a TDPA standard under identical instrumental conditions.

Recovery Studies

Most of the recoveries were conducted with lard products obtained from local retail markets. These samples included both stabilized and unstabilized varieties. Preliminary analyses for antioxidants in these products were conducted to establish that they were free of the compounds used in the recovery studies. Two ml aliquots of the standard solutions (0.2 mg/ml) were pipetted into the distillation flask containing 4 g of melted lard. The ether was then removed by evaporation on a steam bath under a stream of nitrogen. In some instances, all six of the above antioxidants were added to the lard in each recovery study. The analysis was then carried out as previously described.

Results

The recoveries of six antioxidants added at a level of 100 ppm to 4 grams of lard are summarized in Table 1. Recoveries ranged from 84 to 104% for these compounds.

Representative ultraviolet absorption spectra obtained for the antioxidants (BHT, BHA, PG, Ionox-100, and THBP) added to and recovered from lard are shown in Figs. 3 and 4. The infrared spectrum of the methoxylated TDPA recovered from lard is shown in Fig. 5. These spectra demonstrate that the antioxidants can be readily recovered and their identification confirmed by the aforementioned techniques.

Studies have been conducted to determine the ultimate sensitivity limits of the method for the antioxidants recovered from lard with respect to their detection by gas chromatography and confirmation by ultraviolet and infrared spectrophotometry. With this method, BHA and BHT can be detected by gas chromatography at about 1 ppm; however, their identities cannot be confirmed. For quantitation and definite confirmation of the antioxidants in the lards used in this investigation it was established that the lower limits of sensitivity range from 10 ppm (BHA and BHT) to 25 ppm (PG, Ionox-100, THBP, and TDPA). At the present time, NGDA and DLTDP can only be determined qualitatively with this method. Studies are now in progress to investigate the parameters necessary for their quantitative determination.

The procedure has been applied to the analyses of various commercial lards for the presence of BHA, BHT, and PG. The results are tabulated in Table 2. Brand A was

Table 1. Recovery of six antioxidants added to 4 g of lard at a level of 100 ppm

Antioxidant	No. of Runs	Recovery Range (%)	Av. %
BHA	10	88-104	95
BHT	11	85-101	91
Ionox-100	5	89-98	93
PG	11	86-102	96
THBP	9	87-101	93
TDPA	3	84-98	92

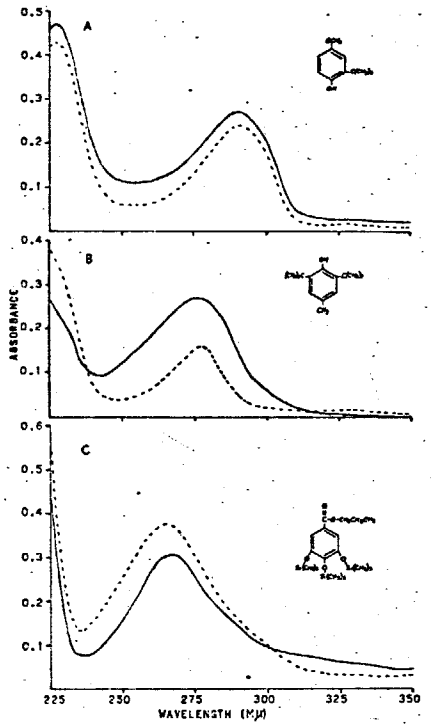

Fig. 3—Ultraviolet absorption spectra of BHA(A), BHT(B), and silanized PG(C), isolated from lard; solid line, unknown; broken line, reference.

found to be the most heavily stabilized; however, the antioxidant content of all of the samples analyzed are below the permitted levels.

Discussion

All of the antioxidants employed in this investigation were effectively and quantitatively separated from the lard under the conditions prescribed. Approximately 1% of the starting material (lard) migrates to the cold finger, thus contributing to the background interferences noted in the preliminary gas chromatographic work. These interferences have since been reduced by the use of a baffle system built into the column wall (Fig. 1). Significant losses have been encountered when the sublimate is accidentally scraped from the cold finger as it is removed from the assembly. This was

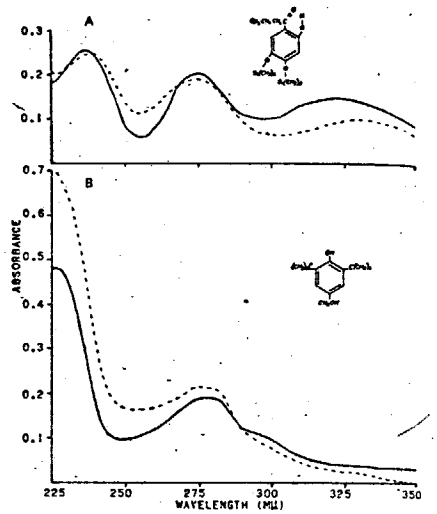

Fig. 4—Ultraviolet absorption spectra of silanized THBP(A) and Ionox-100(B) recovered from lard; solid line, unknown; broken line, reference.

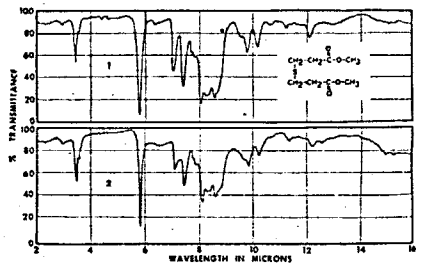

Fig. 5—Infrared spectra of methoxylated TDPA recovered from lard: curve 1, standard; curve 2, unknown.

Table 2. Antioxidants (ppm) found in commercial lards

Brand	BHA	BHT	PG
A	80	82	40
B	0	0	25
C	75	77	37
D	35	0	25
E	26	23	0
F	0	0	37
G	86	0	25
H	40	0	0

corrected by reducing the overall diameter of the cold finger by 2 mm.

Preliminary investigations showed that direct chromatography of the ether solution

for PG and THBP was unsuccessful. Therefore, silyl ether derivatives were prepared; they could be chromatographed because of their increased volatility. Langer, *et al.* (15) first described the use of trimethylsilyl (TMS) compounds to prepare volatile derivatives of phenols for gas chromatographic analysis. However, their method required long reflux times for complete reactions. In our study, the TMS derivatives of PG and THBP were formed by the method of Sweeley, *et al.* (16), using pyridine, hexamethyldisilazane, and trimethyl chlorosilane; this reaction was rapid at room temperature and the analyses can be made within a few minutes.

TDPA also could not be chromatographed directly and attempts to utilize the silanization reaction gave anomalous results. Later studies revealed that the methoxylation reaction with diazomethane could be used to form the dimethyl ester of the acid which can be chromatographed effectively and determined quantitatively.

Mass spectrometry was employed to verify the formation of both the silanized and methoxylated antioxidant derivatives. Mass spectral data showed that silylation was essentially quantitative for BHA, PG, Ionox-100, and THBP (which formed the disilyl ether); BHT remained unsilanized.

In the quest for a quantitative multiple detection method for antioxidants, the following column packing materials were explored: SE-52, SE-30, SE-550, Apiezon L, Carbowax 20M, SAIB, JXR, Epon 1001, DEGS, neopentyl glycol succinate, DC-QF-1, and Versamide 900. BHA, BHT, and Ionox-100 chromatographed well on almost all of these liquid phases. If these compounds are the only antioxidants to be determined, a fast, low-temperature vacuum distillation cleanup (about 20 minutes, 150°C) has certain advantages, particularly with foods of high fat content, since direct extraction techniques remove large amounts of fatty substances along with the antioxidants. These interfering materials not only result in a multiplicity of peaks, but also tend to overload and finally block the column.

Good recovery values were obtained with BHA, BHT, and Ionox-100 when they were vacuum distilled for 45 minutes at 200°C, followed by injection into a 4′ column containing 7.5% GE-XE-60 as the liquid phase. Unfortunately, silanized PG and THBP derivatives were not adequately separated from interfering lard peaks. Recovery ranges were as follows: 86–107% for BHA, 86–104% for BHT, and 87–103% for Ionox-100. The parameters were essentially the same as those previously described for the 16′ column with the exception of the temperature program. In this study the column was heated to 75°C and run isothermally for 2 minutes. Temperature programming was initiated at a rate of 10°C per minute to 150°C, then continued to a maximum of 250°C at a rate of 5°C per minute.

Silanized PG and THBP were separated poorly on most of the liquid phases investigated, and interferences were also encountered from the silanized lard. When prepared as a 16′ column the 3% GE-XE-60 packing material yielded the best overall results by far of any of the phases screened to date.

The response of the flame detector to the antioxidants used in this study was found to be reasonably uniform. Once the operating parameters were determined they were maintained and no attempt was made to readjust to optimum response each day. Therefore, standards were run with each determination. Figure 6 presents the retention time

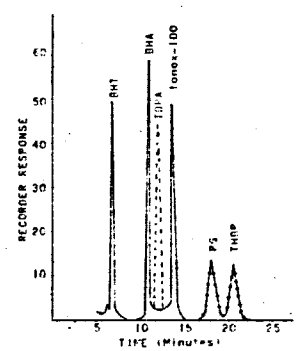

Fig. 6—Gas chromatogram showing typical retention times and responses for six antioxidant standards; ———, *Program I*; —|—|—, *Program II*, and — — —, *Program III*.

of the compounds and their respective responses when 20 μl are injected into the 16′ column at a concentration of 0.2 mg/ml.

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MEAT AND MEAT PRODUCTS

Extraction of Lipids from Raw Beef Lean by Using Various Solvent Systems

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Study shows that chloroform-methanol extraction to remove lipids from beef yielded percentages of total fat equal to or greater than those obtained by the AOAC method. The ratio of phospholipid to triglyceride was always higher in the chloroform-methanol extracted samples. Six extraction method-solvent system combinations, three drying procedures, and two sample preparation lipids were separated by thin layer chromatography. The ratio of phospholipid to triglyceride was calculated after the spectrophotometric determination of the ester groups present. Acid-hydrolysis-Rohrig gave the lowest yield of total lipid and of phospholipid. Sample preparation method or drying method caused few significant differences in the proportions of total solid-

Lipid analyses now include hydrogenation and ozonolysis of triglycerides (TG), phospholipids (PL), individual fatty acids, or other components to provide more information about the effects on fats of cooking, autoxidation, and other conditions. As increasingly elegant methods are developed for analysis of the components of these lipid fractions, involving infrared studies and thin layer and gas chromatographic separations, it has become necessary to re-examine the methods for extracting total lipids from foods.

Many chemists begin their studies of lipid components with a survey of extraction methods or solvents (1–3), whereas others use the chloroform-methanol extraction procedure as outlined by Folch, *et al.* (4) or by Bligh and Dyer (5). However, the standard methods of continuous extraction with ethyl or petroleum ether, or of hydrolyzing the foodstuff with acid to free "bound" lipids before extracting, no longer are considered entirely satisfactory.

In the research reported here, one food, raw beef lean, was used in an attempt to learn whether several extraction methods and solvents yield different amounts of extracted lipids and ratios of PL to TG. We also studied the effects on lipid extraction of two different sample preparation methods and three methods of drying before solvent extraction.

Experimental

Samples and Reagents

(a) *Beef.*—Three cuts of boneless beef round varying in visible fat content were used for this study.

(b) *Acids and organic solvents.*—Reagent grade hydrochloric acid, acetic acid, and organic solvents.

(c) *Mixed ethers.*—Mix equal volumes petroleum ether, b.p., 37.8–46.4°C, and anhydrous ethyl ether.

(d) *2′,7′-Dichlorofluorescein.*—0.2% in methanol.

(e) *Hydroxylamine hydrochloride.*—4% (w/v) in ethanol.

(f) *Sodium hydroxide.*—8% (w/v) in ethanol.

(g) *Basic hydroxylamine reagent.*—Mix equal volumes hydroxylamine hydrochloride and NaOH solutions. Prepare fresh daily.

(h) *Stock ferric perchlorate.*—Dissolve 5 g in 10 ml 70–72% perchloric acid, and dilute to 100 ml with ethanol.

(i) *Ferric perchlorate reagent.*—Dilute 4 ml stock ferric perchlorate + 3 ml 70–72% perchloric acid to 100 ml with cold ethanol. Prepare fresh daily.

Procedures

After the separable fat was removed, lean beef was ground in an electric meat grinder with a divider attachment which partitioned the meat into two equal portions. Grinder plate openings were 5 mm in diameter. One-half of each sample was re-ground twice; the other half was blended in a heavy-duty stainless steel blender. Subsamples of the prepared beef lean were wrapped in aluminum foil and stored in heavy plastic bags or containers at −20°C. Two replications were made. For each assay period, subsamples were weighed to yield 0.2–0.3 g of extracted lipids. Duplicate subsamples were taken for each solvent. Of these, some subsamples were dried in a forced-air oven at 95°C for 5 hr, some were dried in a vacuum oven at 50°C for 48 hr, and others were freeze-dried for 16 hr. Temperatures for the vacuum and air ovens were kept below those recommended by AOAC (6) or by the American Oil Chemists' Society (7) to avoid unnecessary weight loss by removal of volatiles. The shorter drying time in the air oven more nearly resembles conditions recommended by AOAC for the preparation of meat samples for fat extraction.

A pharmaceutical-type freeze dryer was used for subsamples from Cuts I and II. (Sample size was the same as for the vacuum and air ovens.) A pharmaceutical-type freeze dryer was not available for Cut III, which weighed four times as much as the other cuts, and the subsamples were dried in a Dry-Ice type freeze dryer and then ground through a 20-mesh screen in a Wiley mill. From this dried ground meat, smaller portions estimated to contain 0.2–0.3 g of lipids were weighed for extraction.

Soxhlet extractions were made on duplicate dried samples from each drying method with ethyl ether, petroleum ether, mixed ethers, or chloroform; samples were extracted for 16 hr. On the same days, duplicate subsamples that had been stored frozen were scheduled for chloroform-methanol and acid-hydrolysis-Rohrig extractions. Dried samples are not required for the latter two procedures.

Chloroform-methanol extraction was made according to the method of Bligh and Dyer

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INHIBITION OF TUMOUR GROWTH WITH ANTIMETABOLITES OF HEXOSE MONOPHOSPHATE PATHWAY INTERMEDIATES

M. B. SAHASRABUDHE, M. V. NARURKAR and L. B. KOTNIS *

An ideal chemotherapeutic agent should have maximum action on the diseased tissue and no or little action on the host tissue. Unfortunately however, in spite of the best efforts it has not been possible to obtain a completely safe chemotherapeutic drug. A large number of potent anticancer drugs have been discovered in recent years, but all these damage the normal cells also. The margin of safety between the therapeutic effectiveness and toxicity is extremely narrow. To make matters worse, the malignant cells, particularly those which originate spontaneously, are not really foreign to the host. They are a part of the host. The malignant cells therefore, are unable to elicit immunochemical response. In the control of malignant growths, unlike chemotherapy of bacterial diseases, it is not merely sufficient to use cancerostatic drugs and leave the final moping off of the system free of diseased cells, to the defence mechanism of the body. We have to use carcinolytic drugs and the treatment has to go on till the last cancer cell is wiped out. This is obviously a tall order and involves continuation of treatment over protracted periods. Under the circumstances, it is all the more imperative that the drug has to be absolutely non-toxic and should cause no harmful side-effects even after prolonged administration. We have tried to evolve such drugs and some of our results are reported in this communication.

A drug will be effective and at the same time non-toxic, if it interferes with a property which is specific for tumours and which constitutes an essential feature for its maintenance. Unfortunately however, inspite of the sustained efforts, no clear cut differences between the normal and malignant tissues have yet come to light. The differences so far discovered are all of a quantitative nature. Two of these are (1) the low pyridine nucleotide (PN) levels and (2) acceleration of hexose monophosphate (HMP) pathway in the tumour tissue. Rapid HMP results in accelerated production of ribose-5-phosphate which is the starting material for the biosynthesis of purines, pyrimidines and nucleic acids. Acceleration of HMP thus appears conducive to uncontrolled growth. Our recent studies on mechanism of carcinogenesis seem to suggest that efficient operation of HMP is an essential condition for the induction and maintenance of malignancy. Further since the operation of HMP in normal tissue is much less than in malignant tissue, interference of HMP was anticipated to damage malignant cells preferentially and leave the normal cells comparatively untouched.

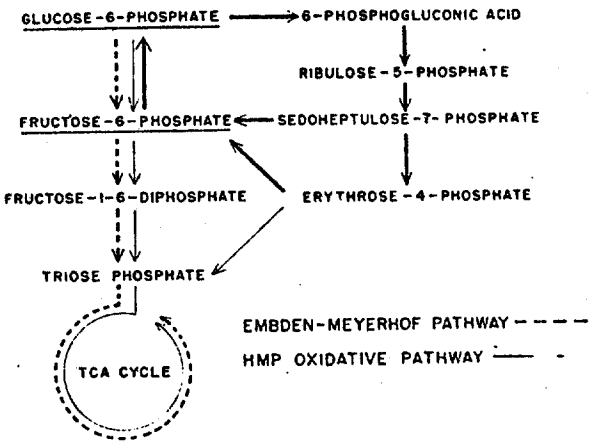

Fig. 1.

Figure 1 shows the Embden-Mayerhof (E.M.) and HMP pathways for the metabolism of carbohydrates. From among the intermediates of HMP pathway only antimetabolites of 6-phosphogluconic acid, sedoheptulose-7-phosphate and erythrose-4-phosphate were considered. Antimetabolites of ribose-5-phosphate, glucose-6-phosphate and fructose-6-phosphate were not considered because these would interfere with E.M. and other normal pathways also and may prove to be toxic.

6-phosphogluconic is known to exist predominantly as a gamma-lactone. There is some evidence that enzymatically produced 6-phosphogluconic acid may be present as a delta-lactone. It was therefore decided to prepare antimetabolites of

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both the gamma-lactone and also the delta-lactone. Figure 2 shows the possible structures suggested

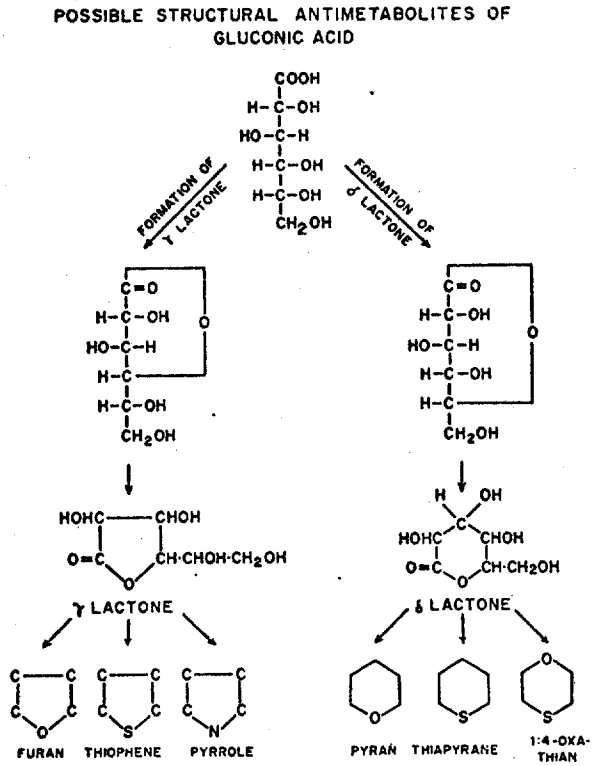

Fig. 2.

TABLE I

Anticancer properties of furan and pyrrolidine derivatives

Name	Yoshida sarcoma ratio of survival E/C	Fibrosarcoma ratio of tumour WTS C/E	Activity
3. 3:4 Bis (Hydroxy)-2:5 Bis carbethoxy-Furan	1.0	1.0	Inactive
4. 2:5 Bis (Carboxy)-N-Phenylpyrrolidine	1.2	1.4	Inactive
5. 2:5 Bis (Hydroxy-Methyl)-N-Phenyl-Pyrrolidine	1.0	1.2	Inactive

TABLE II

Anticancer properties of 2:5 substituted thiophenes

Name	Yoshida sarcoma ratio of survival E/C	Fibrosarcoma ratio of tumour WTS C/E	Activity
6. 2:5 (bis) dihydroxy-Methylthiophene	1.2	1.3	Inactive
7. 2:5 (bis) carboxaldehydethiophene	1.0	1.0	Inactive
8. 2:5 (bis) dicarboxy-thiophene	3.0	2.6	Active
9. 2:5 (bis) mercapto-methylthiophene	2.0	1.5	Active/ Inactive
10. Thiouronium compound of 2:5 (bis) chloro-methylthiophene	>40	2.0 Toxic by S.C.	Active
11. Dihydrazide of Thiophene 2:5-Dicarboxylic acid	1.2	1.4	Inactive
12. 2:5 diacetoxymethyl thiophene	1.0	1.3	Inactive
13. 2:5 dicarbmethoxy-5-dioxytetrahydro-thiophene	1.7	1.4	Inactive

for the proposed antimetabolites. Furan, pyrrole and thiophene structures were considered as antimetabolites for gamma-lactone, whereas thiapyran and oxathiane derivatives were considered for preparing antimetabolites of delta-lactone. 25 compounds were specially synthesized in this series and their properties against transplantable Yoshida sarcoma (ascites) in rats and solid fibrosarcoma in Swiss mice investigated. Yoshida sarcoma is a very rapidly growing tumour and kills the animals on the 4th or at the most on the 5th day after transplantation. The compounds to be tested were usually injected intraperitoneally

24 hours after the transplantation of the Yoshida sarcoma (ascites) cells. Solid fibrosarcoma is a comparatively slow growing tumour ; it kills the transplanted animals by about the 25th day. In the chemotherapeutic screening, the compounds were injected subcutaneously near the tumour mass. The treatment was started on the 6th or 7th day after transplantation. Some of the results are shown in Tables I, II, III, IV, V.

It will be seen that the few compounds synthesized with 6 membered heterocyclic rings

TABLE III

Anticancer properties of 3:4 substituted 2:5 dicarboxy thiophene derivatives

Name	Yoshida sarcoma ratio of survival E/C	Fibrosarcoma ratio of tumour WTS C/E	Activity
14. Transtetrahydrothiophene 2:5 dicarboxylic acid	0.9	0.8	Inactive
15. Cistetrahydrothiophene 2:5 dicarboxylic acid	1.0	1.1	Inactive
16. 2:5 dicarbethoxy-3:4 dihydroxythiophene (dicetol)	Toxic by I.P.	7.5	Active
17. 3:4 diethoxy 2:5 dicarboxythiophene	1.0	1.0	Inactive
18. 3:4 (dimethylenedioxy) 2:5 dicarboxythiophene	0.9	0.9	Inactive
19. 3:4 diphenyl 2:5-dicarboxythiophene	1.5	1.0	Inactive
20. Dihydrazide of 3:4 dimethoxy-2:5-dicarbethoxythiophene	1.5		Inactive
21. 2:5 dicarboxy-3:4-dimethylthiophene	1.0	1.1	Inactive

TABLE IV

Anticancer properties of monosubstituted thiophenes and related compounds

Name	Yoshida sarcoma ratio of survival E/C	Fibrosarcoma ratio of tumour WTS C/E	Activity
22. 2-Thiophenecarboxylic acid	1.0	1.1	Inactive
23. 3-Methylthiophene-3'-Methylsuccinic acid	1.0	1.1	Inactive
24. Methylsuccinic acid	1.2	1.0	Inactive
25. N-Dimethylaspartic acid	1.2	1.4	Inactive

TABLE V

Anticancer properties of open chain compounds

Name	Yoshida sarcoma ratio of survival E/C	Fibrosarcoma ratio of tumour WTS C/E	Activity
26. Thiodiglycollic acid	>50	1.7	Active
27. Diglycollic acid	2.5	1.1	Active/ Inactive
28. Thiodiglycol	>45	1.4	Active
29. Thiodipropionic acid	>45	2.5	Active
30. Thiodibutyric acid	1.7	1.0	Inactive
31. Thiodilactylic acid	2.0	1.3	Inactive
32. Evan acid	2.5	1.2	Inactive
33. Sulphonyl bisacetic acid	1.0	0.9	Inactive

and furan and pyrrolidine derivatives were inactive (Tables I and II) against both the Yoshida (ascites) sarcoma, and solid fibrosarcoma. Among the 18 thiophene compounds tested, 2:5 dicarboxylic acid (TDA), 2:5 dimercaptomethyl thiophene (MMT), thiouronium derivative of 2:5 dimercaptomethyl thiophene (TUMMT) and 2:5 dicarbethoxy 3:4 dihydroxy thiophene (DICETOL) were found to be effective against Yoshida (ascites) sarcoma and also against transplantable fibrosarcoma. With the TUMMT treatment for ten days only, the Yoshida sarcoma transplanted animals survived for more than six months without any trace of malignancy. These animals are still alive and are kept under observation. The corresponding controls died by the 5th day after transplantation. Dicetol gave the best results with solid fibrosarcoma. The tumour weights in the treated animals were merely 1/8th of the control group. Dicetol could not be given by intraperitoneal route because it caused toxic reaction.

While studying the metabolism of Dicetol it was suspected that the thiophene ring ruptures, resulting into an open chain compound. Besides this other considerations had suggested that the lower half of the thiophene ring, composed of carbon atoms nos. 2 and 5 with their substituents and the sulphur atom, may be the main reactive centre of the compounds studied. Accordingly open chain compounds such as thiodiglycollic acid (TDGA) and its higher homologues were synthesized and tested for their anticancer properties. It will be seen that thiodiglycollic acid (TDGA), thiodiglycol (TDGOL), and thiodipropionic acid (TDPA) showed good anticancer properties. These three compounds were able to completely annihilate the Yoshida sarcoma cells with only 10 days treatment. The treated animals survived their natural life span without any trace of malignancy. The corresponding Yoshida sarcoma transplanted control animals died within 10 days (Fig. 3).

In our series we had taken care to see that all our compounds were water soluble. This was necessary to maintain adequate drug concentration at the tumour site to successfully compete with the normal metabolite and to inhibit it from action. That the compounds do interfere with HMP pathway was investigated with the help of glucose labelled at the 1st and the 6th position with C^{14}.

Acute and chronic toxicity studies were carried out with TDA, Dicetol and TDGA; studies with other compounds are now in progress. TDA,

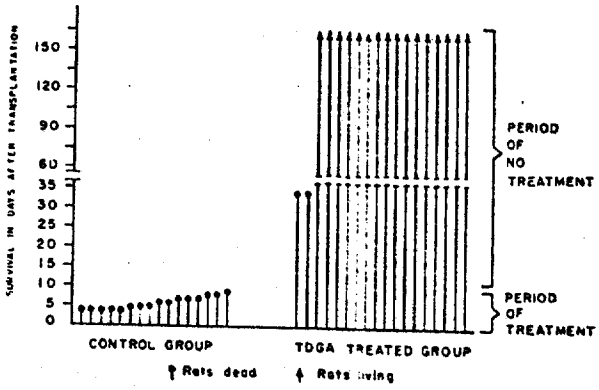

Fig. 3.
Effect of TDGA on the survival of Yoshida sarcoma (ascites) bearing rats.

Dicetol and TDGA could be given 15, 30 and 50 times the daily dose in one instalment without any side effects. Administration of ten times the therapeutic dose for over 15 days did not materially alter the morphology of liver, spleen, kidney or gastrointestinal tract. The only abnormality seen was the complete disappearance of glycogen from the livers of Dicetol treated animals.

Since the margin of safety appeared to be fairly wide, Dicetol and TDGA were tried in advanced and incurable cases. This choice of the clinical material automatically restricted the evaluation of their chemotherapeutic properties. No spectacular results were seen. In three cases of aleukemia Leukemia studied, no response was seen. Other cases studied included malignancies at such sites as breast, lower jaw, head of the pancreas and stomach. In these cases a subjective improvement was seen. Life expectancy was slightly enhanced. The growth of the tumour appeared to be slowed down, pain disappeared and the patient felt better. But it is difficult to say whether this was due to the treatment or some other causes. No toxic side effects were seen and blood picture remained normal even after prolonged administration of compounds.

SUMMARY

Eight of the 38 compounds synthesized by us showed good anticancer properties with no side effects. With four compounds it was possible to bring about complete regression of transplanted Yoshida sarcoma cells. Such spectacular results however were not obtained with solid tumours. One of the reasons may be the poor vascular supply of tumour mass resulting in a poor contact of the drug with malignant cells. Ex-

periments are already underway to get over this difficulty and to screen these and many other compounds which have since been synthesized against a variety of tumours, both spontaneous as well as induced.

RÉSUMÉ

Parmi 38 composés synthétisés par les auteurs, 8 se sont avérés posséder des propriétés carcinostatiques sans effets secondaires. A l'aide de 4 de ces composés on a pu obtenir une régression complète de cellules transplantées du sarcome de Yoshida. Un résultat aussi spectaculaire cependant n'a pas été obtenu sur des tumeurs solides. Une des raisons pourrait en être la faible vascularisation de la masse tumorale occasionnant un mauvais contact entre les médicaments et les cellules tumorales. Des expériences sont en cours en vue de surmonter cet inconvénient et de tester les composés déjà synthétisés et d'autres encore vis à vis de différentes tumeurs soit spontanées, soit induites.

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Fetal Resorption in the Rat as Influenced by Certain Antioxidants[1]

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Some prenatal mortality is perhaps a normal phenomenon accompanying gestation in all mammals. Yet there is a paucity of definitive data on total embryonic or fetal wastage as compared with the wealth of information on the pathologic, genetic, physiologic and nutritional causes of prenatal death. Of the literature which deals with this problem in man or domesticated animals, most of it deals with fetal mortality, either as stillbirths or abortions.

In the rat there are many factors that may increase fetal death and subsequent resorption as, insufficient protein (Barry, '20), elevated environmental temperature (Sundstroem, '27; Hsu, '48; MacFarlane, Pennycuik and Thrift, '57), vitamin deficiencies, e.g., biotin (Kennedy and Palmer, '45), pantothenic acid (Nelson and Evans, '46), pyridoxine (Nelson and Evans, '48), pteroylglutamic acid (Nelson, Asling and Evans, '52), tocopherol (Cheng and Thomas, '53) and riboflavin (Nelson et al., '56). Other causes of resorptions are ovariectomy (Haterius, '36), trypan blue injections (Wilson, '54), Lathyrus odoratus feeding (Walker and Wirtschafter, '56) and others. There is also evidence that loss due to resorption is not confined to any particular period of pregnancy but occurs throughout gestation (wild rat, Perry, '46; wild rabbit, Brambell, '48; mouse, Hollander and Strong, '50).

Because the rat resorbs rather than aborts an imperfect conceptus, a record of all implantations is retained in the uterine mucosa at the implantation sites. This record is represented by remnants of implantation ranging from resorbed fetuses to inconspicuous cicatricial plaques. A careful macroscopic examination of the incised term uterus will therefore supply data on total implantations as well as normal young.

Resorption data secured from the examination of nearly 500 pregnant rats involving approximately 5,000 fetuses and over 500 resorptions is herewith presented. In addition, we investigated the influence of certain maternally administered compounds, mostly antioxidants, on the incidence of fetal loss as compared to normal animals held on normal diets. These data are the outgrowth of a larger problem concerned with the possible value of these antioxidants and related compounds in the prevention or amelioration of lesions arising from neonatal anoxia.

MATERIALS AND METHODS

Walter Reed-Carworth Farms strain rats in their first gestation were used exclusively and weighed approximately 200 gm at time of breeding. After positive mating the females were randomly distributed into experimental and control groups and the former were given *per os* or directly into diet one of the various compounds listed in table 1. Twenty-two days after positive mating the pregnant rat was killed in 100% nitrogen and after 21 minutes the young delivered by caesarian section. Both horns of the uterus were completely incised and a careful macroscopic survey made for resorption sites. The resorptions were charted as to location in the respective region of the horns and classified as to degree of resorption. Seasonal variation in the number of resorptions were recorded.

RESULTS

In 126 normal untreated gravid animals 40.8% had one or more resorptions and 10.6% of all recognizable implantations had terminated in resorptions. Of the 29 dietary regimens used, animals receiving

[1] Supported by U.S.P.H.S. research grant B-1838 and United Cerebral Palsy research grant R-117j-F58.

TABLE 1

Effect of antioxidants and other compounds on fetal resorption[1]

	Total dose	Per os or in diet	Litters	Implantations	Normal fetuses	Litters with resorptions	Resorptions
	gm					%	%
Butylated hydroxytolulene (BHT)	0.5	D	11	124	121	18.2	2.4
d-l,alpha tocopherol acetate	0.2	O	12	169	162	41.7	4.1
2,5-di-tert-amylhydroquinone	0.25	D	10	134	128	60.0	4.5
d gamma tocopherol	0.2	O	11	127	121	45.5	4.7
Butylated hydroxyanisole (BHA)	0.5	D	12	145	137	51.7	5.5
d gamma tocopherol	0.07	O	12	157	148	50.0	5.7
2,6-di-tert-butyl-4-methyl-phenol (DBMP)	0.5	D	11	140	131	54.5	6.4
Thiodipropionic acid	0.5	D	10	128	119	60.0	7.0
1,2-di-hydro-2,2,4 trimethyl-6-ethoxyquinoline (Santoquin)	0.25	D	12	147	136	33.3	7.5
1,2-di-hydro-2,2,4 trimethyl-6-phenylquinoline (Santoflex B)	0.25	D	11	139	128	54.5	7.9
Nordihydroguaiaretic acid (NDGA)	0.5	D	8	86	79	75.0	8.1
Pangamic acid sodium (vit. B_{15})	0.25	D	11	138	126	27.2	8.7
Thiamine hydrochloride	2.0	O	13	160	145	69.2	9.4
Ascorbic acid	2.0	O	14	146	132	71.4	9.6
Glucose	30% of diet	D	25	280	251	68.0	10.3
Normal			126	1430	1278	40.8	10.6
Thiamine hydrochloride	0.5	D	8	75	67	62.5	10.7
2,2-methylene bis(4-methyl-6-tertiary butyl phenol)	0.25	D	8	101	90	37.5	10.9
Polymerized 1,2-dihydro-2,2,4-trimethyl quinoline (Flectol H)	0.25	D	17	148	136	52.9	12.1
Cytochrome C							
Glutathione	0.05	D	8	84	73	50.0	13.1
d-l,alpha tocopherol acetate	0.25	D	6	65	56	66.6	13.9
Ascorbic acid	0.1	O	14	150	129	71.4	14.0
N,N'-diphenyl-p-phenylene-diamine (DPPD)	0.5	D	20	235	199	70.0	15.3
Phenothiazine	0.5	D	23	275	233	60.8	15.3
2,2-methylene bis(4-ethyl-6-tertiary butyl phenol)	0.25	D	10	89	75	70.0	15.7
n propyl gallate	0.25	D	9	111	92	66.6	17.1
Paraminobenzoic acid (PABA)	0.5	D	9	93	76	77.7	18.3
Hydroquinone	0.5	D	9	105	80	77.7	23.8
Methylene blue	0.5	D	10	105	77	100.0	26.8
	0.5	D	10	100	62	90.0	38.0

[1] For samples of antioxidants we are indebted to the American Cyanamid Company for 2,2-methylene bis (4-methyl-6-tertiary butyl phenol) and 2,2-methylene bis(4-ethyl-6-tertiary butyl phenol), Distillation Products Industries for dl-alpha tocopherol acetate and gamma tocopherol, the B. F. Goodrich Company for DPPD, the Monsanto Chemical Company for Santoflex B, Santoquin and Flectol H and Hale Laboratories for vitamin B_{15}.

N,N'-diphenyl-p-phenylene-diamine (DPPD)	0.5	D	23	275	233	60.8	15.2
Phenothiazine	0.25	D	10	89	75	70.0	15.7
2,2-methylene bis(4-ethyl-6-tertiary butyl phenol)	0.25	D	9	111	92	66.6	17.1
n propyl gallate	0.5	D	9	83	76	77.7	18.3
Paraminobenzoic acid (PABA)	0.5	D	9	105	80	77.7	23.8
Hydroquinone	0.5	D	10	105	77	100.0	26.8
Methylene blue	0.5	D	10	100	62	90.0	38.0

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one of 15 showed less resorptions (range 2.4–10.1%) than the controls, while rats receiving one of the other 14 diets showed an increased percentage of resorptions (10.7–38.0%) (table 1). In the dosage given none of these compounds appeared to be toxic to the mother with the possible exception of methylene blue, where poor pregnancy weight gain was noted. This compound yielded, incidentally, the highest percentage of resorptions (38.0%). Of particular interest is the observation that two of the widely used commercial antioxidants, butylated hydroxytolulene (BHT) and butylated hydroxyanisole (BHA), as well as the antioxidant vitamins alpha and gamma tocopherol, at 200 mg level, and 2,5-di-tert-amylhydroquinone gave the lowest percentage of resorptions.

Data on the distribution of resorption sites in all groups revealed that 47.6% of all resorptions were located in the cranial third of the horns, 20.6% in middle third and 31.8% in the caudal third. Most of the sites seen in this cranial region were small inconspicuous cicatrixic plaques suggestive of an early embryonic death.

Using the classification of Walker and Wirtschafter ('56) most of the resorptions, regardless of dietary supplement, belonged either to grade 3, representing complete resorption of the embryo but with small placenta still present, or grade 4, which are cicatrized uterine plaques marking previous placental sites. Grade 2, consisting of small macerated recognizable fetuses, were seldom encountered. The number of fetuses falling into grade 1, e.g., dead, but fully formed, could not be determined since many of our fetuses were killed by anoxic treatment of the mother.

DISCUSSION

Since our observations dealt with the identification of placental sites *in situ*, the question might be raised if identifiable plaques form at all implantation sites. That such scars do form where embryos have been resorbed is well documented by the work on rats of Davis and Emlen ('48). They reported more scars present than young born, the excess being ascribed to resorbed embryos. They also noted that scars do not occur spontaneously in nonpregnant rats. Moreover, these authors and Conaway ('55) reported that the resorption sites may remain identifiable for at least a year in the rat, and Deno ('41) made the same observation on the mouse. Therefore our use of first gestation animals avoided the possible error of counting sites of earlier pregnancies and ascribing them to the gestation under study.

The range of reported resorptions in normal rats by other investigators (11.8–6.5%) (table 2) may be due to seasonal variations in the fertility of the animals. It is generally accepted by rat breeders that fertility is lowest in winter and highest in summer (Main, '39). Since our data was collected throughout the year under controlled environmental conditions, we were able to separate our findings on a seasonal basis. We noted a reduction in the percentage of resorptions during the spring and summer (8.1%) and a marked increase (20.7%) during the winter and fall. Frazer ('55) using Wistar albino rats reported that 31% of all litters had some prenatal mortality on a yearly average, but that it was lowest in July-September (17%) and highest from October to December (86%). Other workers failed to present their prenatal mortality data on a seasonal basis, therefore an accurate comparison of animal groups is difficult, if not impossible.

Another variable influencing the percentage of resorptions in the rat may be the strain difference. Data on 6 different strains, including the wild rat, are summarized in table 2 and, with the exception of our WR-CF animals (10.6%) and Mason's O.M.–Mc. rats (11.8%) the 4 remaining groups agree rather closely on resorption percentages (6.6–8.7%). Yet the strain of the animal may be important, for the findings of Fekete ('47) in the mouse that fertilized ova transplanted to different strains of mice have a significantly better chance of survival in one strain than another, implies that one strain provides a better environment for implantation and development of the ovum. Hollander and Strong ('50) also noted significant differences in fetal mortality among various strains of mice. It is certainly possible that in the rat also, strain differences may exist which could

TABLE 2

Prenatal mortality in mammals

Animal	Litters	Implantations	Normal fetuses	Resorptions	Corpora lutea	Total prenatal mortality	Reference
				%			
Striped hamster	29	193	119	38.3			Fortuyn, '29
Stoat	12	108	103	4.6	135	23.7	Deanesley, '35
Grey squirrel	9		27		30	10.0	Deanesley and Parkes, '33
Sheep	80	109	101	7.2	116	12.9	Hammond, '21
Pig	22	316	267	15.5	396	32.5	Hammond, '21
Pig[1]	495	3528	3485	1.2	4480	22.2	Corner, '23
Pig	102	1117	1028	8.0	1396	26.1	Crew, '25
Rabbit (wild)	68	350	340	2.9	390	12.8	Hammond, '21
Rabbit (wild)	1819		8877		10574	16.0	Brambell, '48
Rabbit (wild)	2179	11363			12551	9.4	Brambell and Mills, '47
Rabbit (wild)	1834	9658	8927	7.6			Brambell and Mills, '48
Rabbit (tame)	56	441	391	11.3	578	32.3	Hammond, '21
Mouse	1080	7967	6672	16.3			Hollander and Strong, '50
Mouse[2]	500	3175	2930	7.7			Danforth and deAberle, '28
Rat (wild brown)	131	1238	1135	8.3	1420	20.0	Perry, '45
Rat (OM-Mc)[3]	50	492	434	11.8			Mason
Rat (Wistar)	25	226	211	6.6			MacFarlane et al., '57
Rat (Wistar)[4]	6	57	52	8.7			Wilson, '54
Rat (Long-Evans)	156		998		1498 (ova count)	33.4	Long and Evans, '22
Rat (Long-Evans)[5]	42	447	410	8.3			Nelson et al., '52
Rat (Holtzman)	35	371	347	6.5			Cheng and Thomas, '53
Rat (WR-CF)[6]	126	1430	1278	10.6			Telford, Woodruff, Linford

[1] Omitted from this data were 13 litters with all implanted embryos resorbing.
[2] From data on 500 pregnancies during last third of gestation as compared to 500 litters 1–3 days old.
[3] OM-Mc designates a mixed strain derived from Osborn-Mendel and McCollum piebald strains (data from personal communication).
[4] Reports present resorptions about 4–5% (personal communication).
[5] Pteroylglutamic acid control animals. Current resorptions in Long-Evans rats about 5% (personal communication).
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account for the fairly wide range of prenatal death rates.

Our analysis of the location of resorption sites in the various regions of the uterine horn revealed that 47.6% of all resorptions were found in the cranial third of the horn, with 20.7% in middle third and 31.8% in caudal third, confirming our impression that the cephalic tapered extremity of the horn was, perhaps, an unfavorable location for the developing embryo. This concept is supported by the observation of Fawcett, Wislocki and Waldo ('47) that the overcrowding by the developing mouse ovum in the anterior chamber of the eye inhibited the growth of adjacent implanted ova. However, Frazer's ('55) data on the rat does not favor this idea, for he demonstrated that the percentage resorption was the same in both horns even though different number of ova were implanted in the different horns. Furthermore, the overcrowding of a mouse uterine horn with an unusually larger number of implanted ova did not influence adversely the resorption rate, as shown by Hollander and Strong ('50).

Another variable influencing fetal loss is the maternal age or weight. Frazer ('55) noted in two strains of rats that the heavier the mother, the higher resorption rate. Similar findings had been reported earlier by Hollander and Strong ('50). All of our animals were standardized at approximately 200 gm giving us uniform test animals thus eliminating the possible discrepancies due to age or weight.

Combining the data in table 2 for all groups of the same species, reveals that the tame rat values fall roughly in the middle of the other mammalian data. For example, the tame rat data show that 9.2% of all implantations end in resorptions. Those mammals with a lower percentage of resorptions are, pig (3.7%), stoat (4.6%), sheep (7.2%), wild rabbit (7.3%) and wild rat (8.3%). Those animals suffering on the average greater fetal loss than the rat are, tame rabbit (11.3%), mouse (15.9%) and wild hamster (38.3%). While fetal wastage is probably common to all mammals considerable species variation does exist.

Basic to our study of the value of antioxidants in the reduction of fetal mortality is the question of their mode of action and whether the site of action of the compound is on the fetal or maternal side of the placenta or both. Most of the agents reported here have been previously used in animal experimentation (Bunnell et al., '55; Schwarz, '58; Mertz and Schwarz, '58; Dam et al., '48, '51). From the results of these workers we assumed that the compound could be assimilated into the blood stream and may exert some influence on the developing placenta and might affect fetal growth. Evidence of placental transfer, however, is available only on a limited number of the tested agents, such as glucose (Huggett, '54), α-tocopherol (Neuweiler, '49), thiamine chloride (Slobody, Willner and Mestern, '49), ascorbic acid (Hamil et al., '47) and phenothiazine (Behn, Frahm and Fretwurst, '56). The rate of placental transfer of fat and water soluble vitamins is quite different, with the lipid soluble vitamins being much slower. In fact in the rat the transfer may be so slow that the cord blood content of vitamin A (Lund and Kimble, '43) or E (Straumfjord and Quaife, '46) may, under certain circumstances, be less than in maternal blood. In the rat Mason and Bryan ('40) reported only limited placental transfer of vitamin E derived from a wheat germ oil concentrate. Yet if 250 mg tocopherol is administered to women 4–8 hours before term, the vitamin E level in the cord is raised significantly (Varangot, Chailley and Rieux, '44). By contrast the water soluble vitamins, thiamine chloride (Slobody, Benson and Mestern, '46) and ascorbic acid (Manahan and Eastman, '38; Chow and Okuda, '60) showed consistently higher concentrations in cord than maternal blood, suggesting even perhaps a concentration of the vitamins by the placenta.

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normal untreated controls. Daily 5 mg dosages of α-tocopherol administered to another group of pregnant animals had no beneficial effect on resorption rate, in fact some negative response was noted (14% resorptions).

In addition to high dosages of α- and γ-tocopherol our data suggest some beneficial effects on resorption rate were obtained from 2,5-di-tert-amylhydroquinone, butylated hydroxytolulene (BHT), butylated hydroxyanisole (BHA), 2,6-di-tert-butyl-4-methylphenol (DBMP), thiodipropionic acid, 1,2-di-hydro-2,2,4-trimethyl-6-ethoxyquinoline (Santoquin), 6-phenyl-1, 2-dihydro-2,2,4-trimethylquinoline (Santoflex B), nordihydroguaiaretic acid (NDGA); while methylene blue, hydroquinone, paraminobenzoic acid (PABA), n propyl gallate, 2,2-methylene bis (4-ethyl-6-tertiary butyl phenol), phenothiazine and N, N-diphenyl-p-phenylenediamine (DPPD) all substantially increase resorption rates.

One is impressed from the data summarized in table 2 that the total prenatal mortality in mammals is much higher than might be suspected from the resorption data alone. Brambell ('48) contends in the rabbit, "that the loss between ovulation and full term is not less than 43% of ova ovulated and may be, and indeed probably is, substantially heavier." Data on total wastage in the rat is scanty, limited to the early work of Long and Evans ('22) and later Perry ('45) on the wild rat. These workers reported respectively 33.4% and 20.0% total prenatal mortality while resorption rate was 8.3% for the two strains of rats.

Other mammals follow a similar trend, ranging from an elevenfold increase in total mortality over resorptions in pig (Corner, '23) to less than a twofold increase in sheep (Hammond, '21). Such observations certainly provide a challenge to animal research workers to seek out the causative factors for such an enormous wastage. These factors are legion, but include such environmental disturbances as abnormalities of blood supply of placenta, an unfavorable implantation site, faulty development of the decidua, abnormal environmental temperature and others. Maternal influences may adversely affect normal implantation and development, such as, inanition, avitaminoses, blood dyscrasias, endocrine disorders, infections and the presence of unphysiologic compounds in the blood such as some of the antioxidants herein reported.

SUMMARY AND CONCLUSIONS

1. In 126 untreated normal pregnant rats 40.8% had one or more resorptions and 10.6% of all implantations terminated in resorptions.

2. The cranial third of the uterine horn contained 47.6% of all resorptions, while the middle third held only 20.6% of the total resorptions.

3. The percentage resorptions in the tame rat falls intermediate among the other mammalian groups.

4. Of the maternally administered compounds, the tocopherols (α and γ), BHT, BHA, amylhydroquinone and DBMP appear to have definite prophylactic value in reducing the frequency of resorptions in the rat, while methylene blue, hydroquinone and paraminobenzoic acid markedly increase resorption rates.

5. Possible extrinsic and intrinsic factors that may influence prenatal mortality in the rat are discussed.

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Spinal Cord

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evaporated to dryness, the residue was redissolved in *n*-hexane and made up to a concentration of 0.5 mg/ml menadione.

Duplicate analyses were carried out for both the GLC method and the NF methods.

Recovery.—Recoveries of added menadione standard were conducted with a sample of known menadione concentration. Addition of the standard was calculated to give a final concentration exactly double that of label claim. A 2 μl aliquot was then analyzed by GLC and compared with a series of menadione standards.

Results and Discussion

When NF standard menadione was added to a sample containing a known amount of menadione, the mean per cent recovery and standard deviation was 98 ± 3%.

Nine different samples of pharmaceutical products were analyzed for their menadione content (Table 1). The samples (tablets, capsules, and injectables) were analyzed by the GLC method and the NF methods. The labelled claims were used as the basis for dilutions and final comparison of the two methods. As most manufacturers put in an excess over the claimed amount, the recoveries would be expected to be 100% of claim or higher. The recovery of claim for all samples was 109 ± 2.2% for the NF methods and 113 ± 3.6% for the GLC method.

A "t" test comparison of the data revealed no significant difference between the results obtained by the two methods, and thus the GLC method for menadione was equivalent to the NF methods when used to analyze the above-mentioned pharmaceutical products. The GLC method has a distinct advantage over the NF methods in that the sample processing and analysis time is reduced by approximately half. Also, a 50 mg sample is required for the NF menadione sodium bisulfite injection method, whereas much smaller amounts can be analyzed by GLC.

Table 1. Comparison of GLC method with official NF methods for the assay of menadione (vitamin K_3) in pharmaceutical products (duplicate analyses)

Type of Sample	Declared Potency of Menadione	Chemical Assay (NF)	GLC Assay
Slip capsules	1 mg/cap	1.10	1.02
Tablets with vitamin C	1.34 mg/tab	1.05	0.93
	0.66 mg/tab	0.78	0.72
Multivitamin tablets	0.1 mg/tab	0.01	0.01
Menadione sodium bisulfite tablets	5 mg/tab[a]	5.2	5.4
Injectables	72 mg/10 ml[a]	78	82
	10 mg/ml[a]	11.4	11.3
	5 mg/ml[a]	5.9	6.0
	5 mg/ml[a]	5.5	5.6

[a] Menadione sodium bisulfite potency.

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OILS, FATS, AND WAXES

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Determination of DLTDP and Other Antioxidants by a Modified Sublimation Technique

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A method has been developed for the determination of dilaurylthiodipropionate (DLTDP) and other antioxidants in lard, using a simplified and less complex vacuum procedure than previously reported. Recoveries ranged from 93–100% for DLTDP, 91–96% for BHT, 85–105% for Ionox-100, 90–100% for PG, and 91% for BHA.

In 1967 we published a multideterminative procedure (1) for 3-(2)*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), 4-hydroxymethyl-2,6-di-*tert*-butylphenol (Ionox-100), *n*-propyl gallate (PG), 2,4,5-trihydroxybutyrophenone (THBP), and 3,3′-thiodipropionic acid (TDPA). The method consisted of a vacuum sublimation of the antioxidants and a quantitative determination by gas chromatography. Since then we have attempted to extend the procedure to nordihydroguaiaretic acid (NDGA) and dilaurylthiodipropionate (DLTDP). Unfortunately, vacuum sublimation was not effective in the isolation of NDGA from lard. The inability to recover NDGA is attributed to its reactivity with the lard constituents which apparently occurs during the sublimation procedure. For example, various authors (2–4) have postulated that NDGA may undergo various oxidative polymerization and actual destructive reactions when heated in the presence of lard.

Under present food additive regulations, DLTDP may be used as a direct or indirect food additive in accordance with prescribed limitations. This stabilizer, antioxidant, or oxygen interceptor has been recommended for use in polyolefins, edible oils and fats, and oil- or fat-containing foods. At the present time there are no methods available for the determination of DLTDP in food products. This is probably due to the marked similarity of the compound to fats and the resultant difficulties encountered with conventional isolative procedures. To maintain effective control over good processing operations and ensure adherence to governmental regulations, it was our purpose to develop a quantitative method for the determination of DLTDP in lard in the presence of other allowable preservatives. It was felt that the vacuum sublimation technique might be a feasible approach to circumvent the aforementioned isolative problem.

In the past there has been apparently some reluctance in the use of techniques involving high vacuum systems. This may be attributed in part to the analyst's unfamiliarity with such procedures. In our experience and as found by other workers in the field (5), a high vacuum apparatus may be bothersome to set up initially; however, once in operation it is no more difficult to operate than the usual chromatographic column. In fact the sublimation[1] procedure is often actually less time consuming since it is possible to eliminate many conventional cleanup procedures such as extractions, chromatography, etc.

During this study it was noted that the sublimation procedure could be substantially modified without sacrifice of precision. The modification resulted in the elimination of the extra high-vacuum pump, the separate diffusion pump, ion gauge, and the electronic systems for monitoring the vacuum. The purpose of this report is to describe this simplified and less complex vacuum procedure utilizing inexpensive laboratory equipment to accomplish the task, thus eliminating the need for sophisticated equipment not normally found in analytical laboratories. This method is applicable to the analysis of

[1] We are using the definition of sublimation as proposed by N. D. Cheronis, *Technique of Organic Chemistry, VI. Micro and Semimicro Methods*, Interscience Publishers, Inc., New York, 1954, p. 85, para. 1.

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DLTDP and other antioxidants (BHA, BHT, Ionox-100, and PG) in lard.

METHOD

Apparatus and Reagents

See reference (1), *Apparatus* (b-e, g-i) and *Reagents* (a-i), except dimensions of distillation flask of sublimation apparatus [Apparatus (d)(*1*)] are now: height = 62 mm and o.d. at widest point = 110 mm.

(a) *Vacuum and diffusion pumps.*—Welch Duo-Seal, Model 1400 (Welch Scientific Co., Skokie, Ill.), or equivalent, equipped with diffusion pump, Type VMF-10 (Consolidated Vacuum Corp., Rochester, N.Y.).

(b) *Vacuum gauge.*—Range 0.1 to 0.001 microns.

(c) *Gas chromatograph.*—Barber-Colman Model 5000 with a flame detector and fraction collector (Barber-Colman Co., Rockford, Ill.).

Column and conditions: For DLTDP, pack 6′ × 3 mm column with 1% Apiezon N liquid phase on 60–80 mesh Gas Chrom Q (Applied Science Laboratories, Inc., State College, Pa.); precondition by heating the column for 48 hr at 270°C while maintaining a carrier gas (argon) flow of 30 ml/min. Maintain the following parameters throughout the analyses: injector temperature 340°C; detector temperature 250°C; carrier gas flow rate, 100 ml/min; hydrogen flow rate, 37 ml/min; air flow rate, 500 ml/min; and attenuation range, X2 at 3×10^{-9} amp into 1 mv full scale recorder.

For BHA, BHT, Ionox-100, and PG, use the 16′ × 3 mm column packed with 3% GE-XE-60 on 60–80 mesh Gas Chrom Q and maintain the parameters as described in the previous report (1).

Procedure

Vacuum separation.—Weigh 4 g lard sample into 60 ml flat-bottom distillation flask. Attach flask to glass column containing built-in baffle system and cold finger, and connect sublimation apparatus to vacuum system as shown in Fig. 1(1). Fill cold finger with liquid nitrogen. (Maintain liquid N level during sublimation.) Start vacuum pump and slowly apply vacuum to system. Obtain 1.5×10^{-4} mm Hg vacuum (as measured by suitable gauge attached between cold finger and trap). Lower assembly into insulated bath maintained at 250°C. After 1.5 hr, discontinue vacuum and remove assembly from silicone oil bath.

Remove retaining clamps and cautiously lift [illegible] (*Caution:* Losses occur by accidental scraping of cold finger against walls of column.) Pour off liquid N. Quantitatively transfer sublimate to concentrator tube by washing cold finger with small portions of anhydrous ethyl ether, using 1 ml hypodermic syringe. Adjust volume to 2 ml by evaporating excess solvent under nitrogen in warm water bath. Mix and retain for gas-liquid chromatographic analysis. (The entire solution will be required for the quantitative determination of DLTDP, BHA, BHT, Ionox-100, and PG.)

Gas-liquid chromatography.—Inject 2 μl ether solution of sublimate to be analyzed into column maintained at 270°C. If DLTDP is present, insert 10:1 stream splitter, inject 100 μl aliquot, and collect effluent in 10 ml collection trap cooled with liquid nitrogen. Retain for identification by infrared spectrophotometry.

The procedures for determining BHA, BHT, Ionox-100, and PG, utilizing the modified method, are similar to those presented in the previous report (1) with the following exception: 2 μl of ether solution of sublimate was injected into a 16′ column packed with 3% GE-XE-60 on 60–80 mesh Gas Chrom Q. To identify the antioxidants a 10:1 stream splitter was fitted to the detector and multiple 25 μl injections were made. Ultraviolet, infrared, and mass spectral analyses were used to confirm the identity of these compounds.

Recovery Studies

Most of the recoveries were conducted with lard products, representing both stabilized and unstabilized varieties, obtained from local retail markets. Preliminary analyses for antioxidants in these products were performed to establish that they were free of the compounds used in the recovery studies. Two ml aliquots of the standard solutions (0.2 μg/ml) were pipetted into the distillation flask containing 4 g melted lard. The ether was then removed by evaporation on a steam bath under a stream of nitrogen. In some instances, all 5 of the above antioxidants were added to the lard in each recovery study. The analysis was then carried out as previously described.

Results and Discussion

The recoveries of DLTDP added at a level of 100 ppm to 4 g lard were satisfactory: 87–105% (98% average) for 6 runs, using the previous high vacuum system, and 93–100% (98% average) for 5 runs, using the new simplified technique.

The infrared spectrum of DLTDP recovered from lard shown in Fig. 1 demonstrates the ability to recover and confirm the identification by the above method.

As a result of the successful determination of DLTDP, the modified vacuum distillation procedure was extended to other antioxidants and found to be applicable; *see* Table 1. Average recoveries were 91, 93, 96, and 93% for BHA, BHT, Ionox-100, and PG, respectively.

In the search for a quantitative method for DLTDP the following column packing materials were investigated: XE-60, Apiezon L & M, Carbowax 20M, Dow 550, DC-QF-1, SF-96, DC-11, FFAP, SE-52, Versamid 900, and neopentyl glycol succinate. In most instances the interfering materials from the lard resulted in a multiplicity of peaks which masked the DLTDP. Of the packings tested, only Apiezon N (1% on Gas Chrom Q) gave the necessary separation.

For quantitation and definite confirmation of DLTDP, the lower limit of sensitivity was estimated at 10–25 ppm. Sensitivity limits were also determined for the antioxidants (BHA, BHT, Ionox-100, and PG) and found to be similar to those reported previously (1).

Table 1. Recovery of antioxidants added to 4 g lard at a level of 100 ppm

Antioxidant	No. of Runs	Recovery Range, %	Av., %
BHA	3	91	91
BHT	4	91–96	93
Ionox-100	5	85–105	96
PG	5	90–100	93

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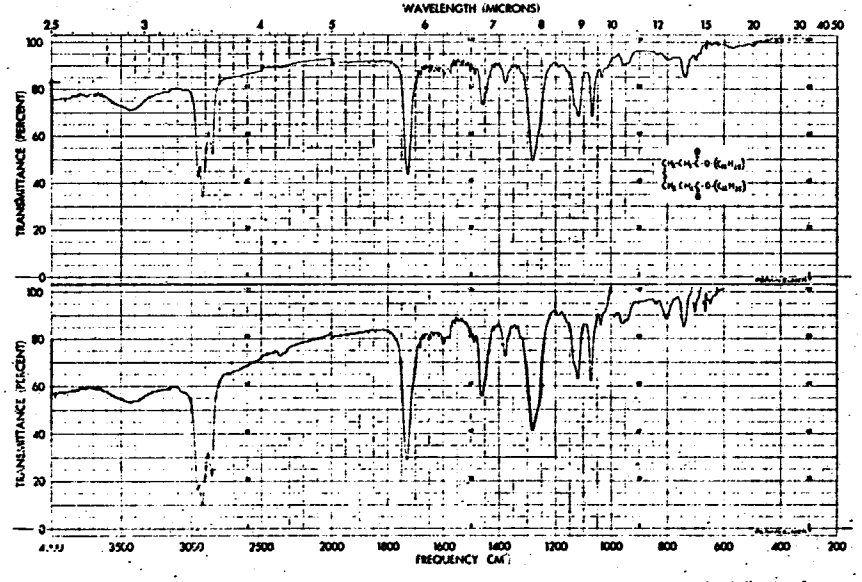

Fig. 1—Infrared spectra of DLTDP standard (top) and DLTDP recovered from 4 g lard (bottom).

study: Eastman Chemical Products, Inc., Kingsport, Tenn.; Shell Chemical Co., Division of Shell Oil Co., Union, N. J.; and Evans Chemetics, Inc., New York, N. Y.

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Determination of Saturated and Unsaturated Fatty Acids: Comparison of Gas-Liquid Chromatographic, Thiocyanogen Number, and Lead Salt-Ether Methods

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The weight per cent composition of the C_{16}–C_{18} series of fatty acids in edible vegetable oils was determined by GLC, lead salt-ether, and thiocyanogen number methods. Total saturated and unsaturated fatty acid percentages obtained by GLC and the lead salt-ether methods were in poor agreement; the lead salt-ether unsaturated fatty acid percentages tended to be higher than the GLC values. The thiocyanogen number values for unsaturation were higher than GLC values for every fat and oil analyzed except one. The polyunsaturated fatty acid percentages obtained by the thiocyanogen number method were not similar to the GLC values for about half of the fats and oils examined.

The fatty acid compositions of fats and oils have been determined by various chemical methods (1) and by gas-liquid chromatography (GLC) (2–6). Only a few papers compare the analyses of oils by GLC with a standard chemical method (7, 8). To our knowledge there is no published data available comparing GLC, lead salt-ether (LS-E), and thiocyanogen number (TN) values in a composite study. The TN method has the limitation that $C^{3=}_{18}$ is determined not directly but rather by difference; thus the presence of fatty acids, other than the C_{18} series, containing double bonds can influence the $C^{3=}_{18}$ estimate. The LS-E method has a number of drawbacks; it is essential that the hydrolysis of the lead salt to lead chloride be complete and that the ether wash be $AgNO_3$-negative to ensure complete removal of the HCl. The determination of the saturated and unsaturated fatty acids by the LS-E method as described in *Official Methods of Analysis* (9) is inherently difficult due to omissions from the original method (10) and from the referee reports (11, 12). Both the TN and the LS-E methods are difficult and time consuming as compared to GLC analyses.

The present study was undertaken to compare, on the same samples, the analyses of the C_{16}–C_{18} series fatty acids found in commercially available edible vegetable oils by the LS-E, TN, and GLC methods. The C_{16}–C_{18} fatty acid series is of special importance because of current interest in saturated: unsaturated and saturated:polyunsaturated ratios of commercial vegetable fats and oils.

Experimental

Reagents and Materials

(a) *Oils.*—The following edible vegetable oils were purchased from local supermarkets: cottonseed, peanut, corn, soybean, safflower, olive (imported), oleomargarine (regular and soft).

(b) *Standard fatty acid methyl esters.*—Applied Science Laboratories, State College, Pa.

(c) *Cottonseed oil.*—USP (Fisher Scientific Co., Pittsburgh, Pa.).

(d) *n-Hexane.*—Pure grade (Phillips Petroleum Co., Bartlesville, Okla.).

Chemical Methods

The total saturated and unsaturated fatty acid weight values were determined by the LS-E method according to the official, final action AOAC method, 26.040 (9). The determination of the saturated and unsaturated fatty acids by the LS-E method as described in *Official Methods of Analysis* (9) is difficult due to omissions from the Baughman and Jamieson method (10) and the referee reports of Jamieson (11, 12). The modifications of the AOAC method employed in this study have been previously reported by Sheppard and LaCroix (13).

Oleic, linoleic, and linolenic acid composition was determined by the official, first action AOAC TN method, 26.026–26.027 (9) with no modifications. The iodine absorption number (Hanus) was determined by the official, final action AOAC method, 26.020–26.021 (9), to obtain iodine absorption numbers for the TN method. All determinations were performed in duplicate.

Gas Chromatography

The fatty acids were determined as methyl esters on a packing of 14% ethylene glycol succinate on 80–100 mesh Gas Chrom P. A Barber-Colman 5000 gas chromatograph was equipped with a 6' × 3 mm i.d. Pyrex column and a high temperature hydrogen flame ionization detector. The electrometer output was 3×10^{-9} ampere full scale deflection with a 5 mv recorder. Argon carrier gas flow rate was 60 ml/min. Operational temperatures were as follows: flash heater 280°C; detector 280°C; column 175°C. Methyl esters of sample fatty acids were prepared by the method of Stoffel, Chu, and Ahrens (14) as modified by Sheppard and Ford (15). The GLC was calibrated in terms of weight response in the following manner: equal weights (1.25 μg/μl *n*-hexane) of individual standard fatty acid methyl esters of hexadecanoate (C_{16}), octadecanoate (C_{18}), 9-octadecenoate ($C^{1=}_{18}$), 9,12-octadecenoate ($C^{2=}_{18}$) and 9,12,15-octadecenoate ($C^{3=}_{18}$) were injected as a mixture into the gas chromatograph. All methyl esters were checked for purity by GLC. Each ester showed one elution peak with no indication of minor peaks being present. Methyl ester peak areas were determined by the standard triangulation method.

The gas chromatograph was calibrated in the following manner: the areas of the various methyl esters were related to the response for the same weight of C_{16}. The weight correction factor equals the area of C_{16} divided by the area of C_x. The corrected response areas (area C_x × weight correction factor C_x) thus may be used to calculate the relative weight percentage of the components in the sample. Weight correction factors and retention times relative to C_{16} are given in Table 1.

Results and Discussion

The major fatty acid composition of the edible oils as determined by GLC are shown in Table 2. The weight per cent distribution of the individual fatty acids and the total fatty acid weight per cent agree well with known standard vegetable oils as determined by GLC (2–7). In addition, the GLC method makes possible the analysis for trace amounts of other fatty acids such as tetradecanoic (C_{14}), 7-hexadecanoic ($C^{1=}_{16}$), eicosanoic (C_{20}), and the eicosenoic series ($C^{1-5=}_{20}$) which cannot be determined by the TN method.

Table 3 shows the percentage composition of the saturated, unsaturated, and poly-

Table 1. Summation of relative retention times and weight correction factors as determined with a 14% ethylene glycol succinate column and a high-temperature hydrogen flame ionization detector

Fatty Acid Methyl Ester	Relative Retention Time	Weight Correction Factor (C_{16} = 1)
C_{16}	1.0000[a]	1.0000
C_{18}	1.745	0.9950
C^1_{18}	2.005	1.0179
C^2_{18}	2.523	0.7766
C^3_{18}	3.390	0.9095

[a] Retention time of 6.7 min.