ACETIC ACID AND ACETATES VOL III #88
COPIES OF ARTICLES CITED

Acetic Acid & Acetates-Volume 3-Copies of Articles Cited #88 2/25/74

VOLUME 3

GRAS MONOGRAPH SERIES

ACETIC ACID AND ACETATES

(COPIES OF ARTICLES CITED IN MONOGRAPH SUMMARY)

prepared for

THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION AND WELFARE

FEBRUARY 25, 1974

This publication was prepared under Contract Number FDA 72-100
with the Public Health Service, Food and Drug Administration,
Department of Health, Education, and Welfare

prepared by

Tracor Jitco, Inc.

S. Afr. J. med. Sci. (1961), **26**, 31-61.

SOME ASPECTS OF THE METABOLISM OF KWASHIORKOR AND OF NORMAL INFANTS AS DETERMINED BY THE UTILISATION OF 1-^{14}C SODIUM ACETATE, 2-^{14}C PYRUVATE AND UNIFORMLY-LABELLED ^{14}C GLUCOSE.

by

JOSEPH GILLMAN*, THEODORE GILLMAN†, JOAN SCRAGG‡, NERINA SAVAGE*, CHRISTINE GILBERT*, GORDON TROUT*, PAUL LEVY*

[RECEIVED 24TH MARCH, 1961]

The prevention of kwashiorkor is no longer a scientific problem. With general improvement of feeding and appropriate education, the disease could be eliminated from countries, including the African Continent, where it is endemic. Unfortunately, until well-planned, national feeding programmes are launched it will still be necessary to treat the florid forms of the syndrome. At the present moment, despite the recommended methods of treatment, the mortality in our hospital may still be as high as 35% and, judging from recently published reports, is not very much different in other parts of Africa and in Jamaica [Collis, 1958; Schendel and Hansen, 1958].

Kwashiorkor, like other diseases, has a natural history. When cases are admitted to hospital, they may be in different stages of evolution, even though the clinical signs may appear to be similar [Gillman and Gillman, 1951; Behar *et al.*, 1958]. Using the response to diet as a criterion for defining the stage of the disease, we recognise two main "types". The first or compensated type responds rapidly, while the second, or decompensated type, fails to respond to good food. Such cases may deteriorate and, despite all forms of therapy, fail to survive. Alternatively, incompletely decompensated infants may linger on and eventually become marasmic from which recovery, if it occurs, is very protracted.

By definition, it is not possible to recognise clinically on admission to hospital the decompensated type of kwashiorkor as this can only be done by taking into account the response to therapy. We can say that, on admission, the livers of the decompensated cases of florid kwashiorkor are intensely fatty, although, if they survive for a variable period without much clinical improvement and become marasmic, the fat in the liver may disappear or be greatly reduced. This explains the variable amount of fat found in the liver in infants dying from kwashiorkor. Clearly, in decompensated kwashiorkor some metabolic disorder has supervened in addition to the original, nutritionally-dependent disturbance and, unless adequately corrected, the infant may die even though fed a satisfactory diet. If, therefore, the high mortality rate which, at present, cannot be controlled by any known form of therapy is to be reduced in the immediate future, it is urgent to gain further information about the nature of the metabolic disorder in the decompensated type of kwashiorkor.

A metabolic disorder can be assessed in different ways, each of which may contribute eventually to an understanding of the key site of the disorder. In clinical and pathological descriptions of kwashiorkor attention has been focussed on the dermatosis,

*Department of Physiology and Joint C.S.I.R./University Nutrition Research Unit, University of the Witwatersrand, Johannesburg.
†Department of Physiology, Faculty of Medicine, and C.S.I.R. Unit for Study of Growth and Ageing, University of Natal, Durban.
‡Department of Paediatrics, Faculty of Medicine, University of Natal, Durban.

the oedema, the fatty liver, the low plasma proteins and atrophy of the pancreas and mucous membranes [Gillman and Gillman, 1951; Trowell *et al.*, 1954].

The mechanism responsible for oedema in kwashiorkor is still enigmatic. The fatty liver is of rapid onset, not immediately dependent on any particular amino acid deficiency [Gillman and Gillman, 1951], and probably the consequence of some acute derangement of the endocrine glands [Gillman and Gilbert, 1958]. The low plasma protein is still generally regarded to-day as due to an insufficiency of first class proteins in the diet and this in turn is said to interfere with plasma protein synthesis, despite the repeated observations that many acutely-developing metabolic disorders may lead to a sharp drop in the plasma albumin. The atrophy of the pancreas, frequently accompanied by the marked reduction or even absence of faecal trypsin, together with the marked atrophy of the mucosa of the stomach and small intestine, have all been taken to mean that the dissimilation and absorption of ingested proteins are impaired. This is now known not to be the case, since balance studies in several independent laboratories have demonstrated that proteins can be dissimilated and effectively absorbed by kwashiorkor infants even a day or two before death [Holemans and Lambrechts, 1955; Waterlow and Wills, 1960]. In these circumstances, therefore, despite the apparent reduction in the enzymes in the stomach, intestines and pancreas, proteins can be dissimilated and the amino acids absorbed efficiently.

Similarly, although considerable data have been accumulated relating to the kinds and amounts of the enzymes present in the blood and tissues of kwashiorkor [Scrimshaw *et al.*, 1956; Burch *et al.*, 1957; Waterlow, 1957, 1958] these do not permit an understanding of the central metabolic disorder. An increase or decrease in an enzyme in itself may have little meaning without relation to the nature of the prevailing metabolism or to a knowledge of the role of that enzyme in metabolism. In other words, the concentration of a particular enzyme may be found to be increased or reduced and yet this changed concentration of the enzyme may only be an epi-phenomenon rather than an expression of the primary metabolic disorder.

The study of the glucose concentration in the plasma or for that matter of other constituents of the blood, such as lipids, electrolytes, vitamin A or other vitamins, has not been particularly helpful in elucidating the nature of the altered metabolism in kwashiorkor, especially when such studies have been statistical (on large groups of infants), rarely serial, and unrelated to the evolution of the syndrome in each case, that is, whether decompensated or compensated.

In approaching the characterisation of the metabolic disorder in kwashiorkor, it seemed necessary to us to attempt to ascertain whether the kwashiorkor infant, both compensated and decompensated, used particular fuels, such as glucose, pyruvate and acetate, in ways different from those of seemingly healthy infants of a comparable age. Since the utilisation of the above-mentioned compounds, thus far investigated, did not only mean oxidation but also utilisation for synthetic processes, (e.g. of plasma protein, cholesterol, phospholipids, triglycerides and glucose) we found ourselves in the position to be able to express some views, inter alia, on whether or not the low plasma protein or low serum cholesterol was due to impaired synthesis or altered utilisation or to a combination of both processes.

The selection of uniformly-labelled ^{14}C glucose, 2-^{14}C pyruvate and 1-^{14}C acetate for these studies in kwashiorkor was to some extent determined by the fact that we had already conducted several studies in baboons subjected to various endocrine ablations and had gleaned much information about the endocrine control of the fatty liver and about the synthesis of the various lipid components of the serum [Gillman *et al.*, 1957, 1958 a, b, c, 1959; Gillman and Gilbert, 1958a, b; Gilbert *et al.*, 1960; Savage *et al.*, 1960 a, b, c]. The rapid development of the fatty liver in kwashiorkor

within 8 days [Gillman and Gillman, 1951] and the absence of fat from the livers of undernourished infants and children not suffering from kwashiorkor, suggested to us that kwashiorkor was an acute metabolic disorder supervening in infants and children with a background of moderately severe malnutrition. The development of the fatty liver during the acute episode, therefore, was more than likely dependent on the altered participation of the endocrine glands [Gillman and Gilbert, 1958a]. Apart from adding some data about the utilisation of pyruvate, acetate and glucose in kwashiorkor, we hoped to obtain indirect information about the role of the endocrine glands in the decompensated infant.

Accordingly, in this paper, we propose to record our observations relating to (1) the oxidation to carbon dioxide of uniformly-labelled ^{14}C glucose, 2-^{14}C pyruvate and 1-^{14}C acetate; (2) the incorporation of ^{14}C into the proteins, cholesterol, fatty acids of triglycerides and phospholipids, glyceride glycerol and glucose of the plasma and/or serum and, where circumstances permitted, also of the liver and other tissues; (3) the removal of glucose from the circulation after administration of labelled glucose; (4) the incorporation of ^{14}C into the plasma proteins during the first 5-7 hours of the experiment and for 10 days thereafter. These several chemical studies will permit us to list some of the major differences in metabolism of compensated and decompensated cases of kwashiorkor, as compared with control infants. Finally, we shall draw attention to a more than likely dependence of the normal infant on dietary fat for cholesterol and fatty acid synthesis, and on carbohydrates as well as fat for the synthesis of proteins of the plasma and possibly also of the tissues.

MATERIAL AND METHODS

Ten subjects were investigated by tracer techniques; 5 were infants suffering from florid kwashiorkor and 5 control infants of comparable age. The main clinical features of the kwashiorkor infants are shown in Table I. As is well known, on the day of

TABLE I
Clinical findings.

Case No.	Age (months)	Sex	Duration of history (weeks)	Oedema ‖	Dermatosis §	Diarrhoea	Anorexia	Mental change ¶	Liver Size**
†K-1	18	F	2	++++	++++	++	++++	+	1-3
†K-2	14	F	>3	++++	++++	+++	++	+	4
‡C-3	22	M	—	—	—	—	—	—	1
†K-4	24	F	1	++++	++++	++++	++++	+	2
C-5	24	M	—	—	—	—	—	—	1
C-6	24	M	—	—	—	—	—	—	1
*K-7	15	F	2	++++	++++	++	+++	+	1½
C-8	13	F	—	—	—	—	—	—	—
*K-9	14	F	2	++++	++++	++	++	+	1½
C-10	17	F	—	—	—	—	—	—	—

C = Control.
K = Kwashiorkor (*Compensated. †Decompensated).
‡ = Admitted as Kwashiokor 51 days previously; clinically recovered.
‖ = Massive oedema.
§ = Gross dermatosis (hyperpigmentation, pseudopurpura, desquamation, depigmentation).
¶ = Marked apathy.
** = Figures indicate number of finger breadths below costal margin in mid-clavicular line.

TABLE II
Laboratory findings.

Case No.	Hb (g.%)	P.C.V. (%)	MC.H.C. (%)	Prothrombin index (%)	Serum bilirubin (mg.%)	Serum urea (mg.%)	Serum cholesterol (mg.%)	Glucose (mg.%)	Total proteins (g.%)	Albumin (g.%)	Globulin (g.%)	Globulin fractions (g.%) α_1	α_2	β	γ	Alkal. phosph. (K.A. units)	$ZnSO_4$ turbidity (units)	Thymol turbidity (units)
†K-1	12·2	37	33	35	0·8	33	136	67	3·8	1·4	2·4	—	—	—	—	11	14	10
†K-2	11·4	38	30	—	<0·8	36	230	131	5·2	1·7	3·5	—	—	—	—	10	18	20
C-3	10·7	34	31	100	<0·8	56	188	81	8·2	2·3	5·9	—	—	—	—	28	24	10
†K-4	11·7	—	—	88	<0·8	36	—	44	4·3	1·1	3·2	0·6	0·7	0·7	1·2	6	8·7	5·3
C-5	11·5	36	32	92	<0·8	31	210	66	7·3	2·6	4·7	0·5	0·9	1·2	2·1	13·7	22	6
C-6	9·0	29	31	—	<0·8	55	124	61	7·8	2·9	4·9	0·7	1·1	1·3	1·8	13	11	2
*K-7	10·8	34	32	94	0·6	19	76	57	5·2	1·4	3·8	0·5	1·1	1·0	1·2	11·2	4	4
C-8	9·2	31	30	86	0·4	33	140	67	8·4	2·7	5·7	0·8	1·1	1·5	2·3	18	16	8
*K-9	8·0	25	32	35	2·0	26	275	83	5·1	2·0	3·1	0·6	0·7	0·8	1·0	8·4	4	17
C-10	12·1	38	32	89	0·46	29	148	81	8·0	2·7	5·3	0·8	1·2	1·5	1·8	2·7	5	3

K = Kwashiorkor (*compensated; †decompensated).
C = Control.

admission to hospital, prognostication, on the basis of one or more clinical criteria, is unreliable, and this proved to be so in our present series of 5 cases of which three were decompensated while two were compensated (Table I). The size of the liver permitted a slightly better prognosis to be made but even then it was not entirely satisfactory.

The various routine laboratory investigations conducted on these infants are shown in Table II. Again it can be seen that no single investigation could be used to predict whether or not the infant would survive. The cholesterol was grossly elevated in a case that died (K-2) as it was in a case (K-9) that recovered. In one instance the cholesterol was 76 mg. % (K-7) but the infant recovered. The blood urea was generally more elevated in our cases than in those reported elsewhere [Dean and Schwartz, 1953]. In all our kwashiorkor cases the concentration of the plasma proteins was invariably reduced.

Some of the infants, controls as well as kwashiorkor cases were fed during the test while others were fed only at the end of the third hour. The latter received no food after 2 a.m., the test beginning at 8.30 to 9 a.m. of the same morning. Feeding did not apparently affect the results. Accordingly in the last 6 cases we resorted to the regimen where the infant was not fed until the 3rd hour after the administration of the tracer.

^{14}C compounds. 2-^{14}C sodium pyruvate (specific activity (S.A.) 1 mc/mM); D-glucose-^{14}C (G) (S.A. 10mc/mM) and sodium acetate 1-^{14}C (S.A. 2 mc/mM)were obtained from the Radiochemical Centre, Amersham. The sealed ampoules were opened and the contents dissolved in 10 ml. normal saline immediately before administration.

Blood was drawn for various analyses at intervals to be indicated in the descriptive part of this study. A liver biopsy was taken between the 5th and 8th hour after the administration of the tracer or, in one instance, 24 hours later. Further liver biopsies were taken in some cases, 6 and/or 10 days later, for histological and radiochemical analysis.

CO_2 collection. The expired air was collected in tubes containing saturated barium hydroxide solution from a mask connected to an appropriate, very low resistance valve. This method of collection was not entirely satisfactory, as we suspected that there was some loss of gas particularly in the case of the more vigorous controls. During the CO_2 collection the vessel containing the barium hydroxide was repeatedly shaken. The $BaCO_3$ was quantitatively collected on to sintered glass crucibles, washed repeatedly with hot water, followed by alcohol and ether and finally dried in the oven at 100°C. The crucibles were then weighed and the weight of barium carbonate determined. The $Ba^{14}CO_3$ samples were converted again to CO_2 by addition of dilute HCl and the CO_2 absorbed into a standard solution of CO_2-free NaOH in an evacuated system. The samples were plated and counted as described previously [Savage *et al.*, 1960 a].

Glucose. Glucose was isolated from protein-free filtrates of whole blood [Somogyi, 1945] as the osazone, according to the method described by Zilversmit *et al.*, [1948].

Total Serum or plasma proteins. The proteins were precipitated in the cold with 20% trichloroacetic acid solution, and treated thereafter according to the method of Manchester and Young [1959]. The final protein precipitate was extracted twice

with a mixture of 4:1 methylal/methanol and finally washed with alcohol:ether (1:1) and ether. The precipitate was dried in a vacuum desiccator.

In order to demonstrate that the radioactivity of the protein in kwashiorkor was not due to ^{14}C-labelled carbohydrate constituents of glyco-proteins occurring in serum or plasma, the protein was hydrolysed with 6 N HCl for 24 hours at 120°C. The hydrolysate was then passed through a Dowex 50 column in the acid form. The column was washed successively with distilled water, 0·3 N HCl, 1 N HCl, 3 N HCl and finally with 4 N HCl. On testing the activity of the various fractions so obtained we found 80-85% of the activity in the fraction eluted with 1 N HCl. Although not conclusive, this would rule out to a large extent the presence of ^{14}C carbohydrate constituents. This matter is being further investigated.

Serum lipids. The triglycerides and lipid phosphorus, as well as the fatty acids thereof, and the cholesterol, were fractionated and the radioactivity determined according to methods described previously [Savage *et al.*, 1960 a, b, c].

Isotope determination. All samples were assayed as $BaCO_3$, after prior conversion to CO_2 by the wet carbon combustion method of Van Slyke *et al.*, [1951]. The procedures for plating and counting were as described previously [Savage *et al.*, 1960 a, b, c].

RESULTS

Specific Activity of the Plasma Proteins

The data relating to the incorporation of the isotope into the proteins of the serum plasma and liver are shown in Table III. Irrespective of the nature of the isotope, i.e. whether pyruvate, glucose or acetate, the specific activity of the plasma serum proteins of the kwashiorkor cases was 2½-7 times greater than the specific activity of the plasma proteins of the controls. Moreover incorporation was greater in the decompensated (K-1, K-2, K-4) than in the compensated cases.

The rate of incorporation of sodium acetate and sodium pyruvate into the plasma protein of 4 cases (2 control, C-10, C-8, and 2 kwashiorkor, K-7, K-9) is shown in Fig. 1. This shows that the specific activity of the plasma proteins in the kwashiorkor cases was 3 times greater at 1½ hours and 4 times greater at 6½ hours after the commencement of the experiment than at corresponding intervals in the control. By the 10th day, the specific activity of the plasma protein in the case of acetate had decreased markedly and was approximately the same for the kwashiorkor infant (K-7) as for the control (C-10).

Where sodium pyruvate was used, the rate of incorporation at the end of one hour was three times as great in the kwashiorkor as compared with the control case; this difference in the rate of incorporation was maintained during the succeeding 3 hours. From Fig. 1 it would appear the incorporation had not reached its maximum either in the control or in the kwashiorkor cases. By the 10th day, radioactivity of the serum proteins had been reduced to about 1/7th of that attained 5½ hours after the administration of sodium pyruvate in kwashiorkor and to 1/40th in the control (Table III).

The incorporation of 1-^{14}C sodium acetate into the plasma proteins was strikingly different in the kwashiorkor case from that in the control. In the former the rate of incorporation was rapid, more extensive and had not reached a plateau even at the end of 6 hours. In the control, apart from the much lower level of incorporation, the plateau was reached as early as one hour.

The extent of incorporation of radioactivity into the plasma proteins of both the kwashiorkor and control cases was not related to the initial concentration of plasma proteins (Table III).

TABLE III

Incorporation of isotope into liver and plasma proteins in kwashiorkor and control infants.

Isotope	Case No.	Time after isotope injection	Specific activity cts./min./mg. Plasma protein	Liver protein
2-^{14}C Pyruvate	*K-9	6 hrs.	44·5	50·9
		10 days	6·9	11·5
	†K-1	7 hrs.	111·7	126·0‡
	†K-2	7 hrs.	101·5	91·0
	C-8	5½ hrs.	17·1	—
		10 days	0·4	—
	C-3	7 hrs.	14·1	Not detected
	C-6	6½ hrs.	17·0	21·8
^{14}C Glucose	†K-4	6 hrs.	64·2	84·1
	C-5	6 hrs.	13·1	12·7
1-^{14}C Acetate	*K-7	6 hrs.	53·8	10·1
		10 days	2·3	2·2
	C-10	6 hrs.	12·1	10·7
		10 days	3·1	4·1

K = Kwashiorkor (†decompensated; *compensated).
C = Control.
‡ = Biopsy at 24 hrs.

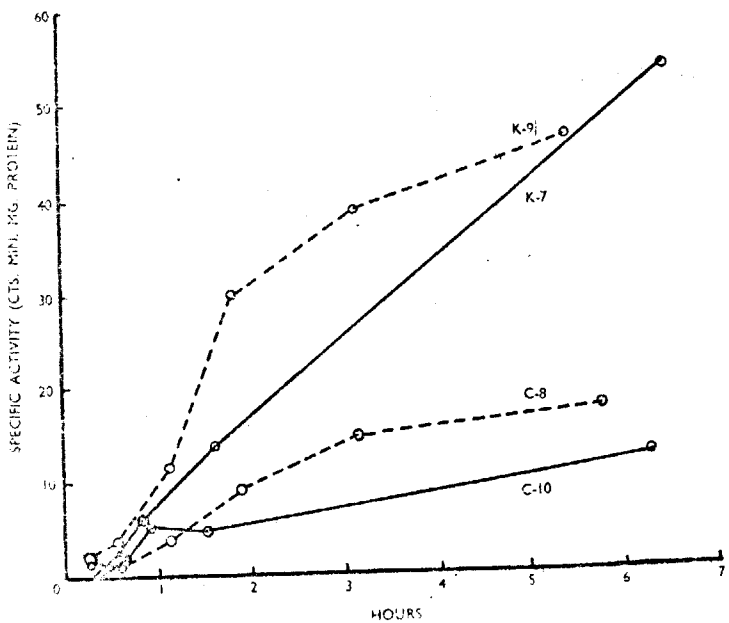


Fig. 1. Rate of incorporation of 2-^{14}C pyruvate and of 1-^{14}C acetate into plasma proteins of kwashiorkor and control cases: interrupted line = 2-^{14}C pyruvate; solid line = 1-^{14}C acetate; C = control; K = kwashiorkor.

Specific Activity of Liver Proteins

Analysis of the specific activity of the proteins of the liver, obtained by biopsy puncture, was undertaken in 8 cases (Table III). With the exception of K-7, the specific activity of the liver proteins corresponded very closely with that of the plasma proteins in each case. In K-7, the specific activity of the liver proteins was one-quarter that of the plasma proteins at the corresponding time after the beginning of the experiment; the poor correspondence may have been due to the possibility that while taking the liver biopsy, more of Glisson's capsule than liver parenchyma was sampled. It is noteworthy that in 3 cases (K-7, K-9, C-10) where liver biopsies as well as plasma proteins were obtained on the 10th day, there was a close correspondence between the specific activity of the proteins of the plasma and liver.

In five cases, a second biopsy was taken between the 6th and 10th day after the beginning of the experiment. In the first decompensated case (K-1) the specific activity had fallen at the end of the 6th day from 126 to 43 cts./min./mg.; in K-2 (decompensated), the specific activity had decreased by almost half within 48 hours and in K-9 (the compensated case) to almost ¼ at the end of 10 days. In K-7, the specific activity of the liver proteins had fallen to ½ and in the control case (C-10) to 40% of the initial value at the end of 10 days.

Specific Activity of Tissue Proteins (Table IV)

Permission was obtained to carry out a post mortem examination on 2 decompensated kwashiorkor infants who died 43 and 158 hours, respectively, after the administration of 2-^{14}C pyruvate. The specific activity of some of the tissue proteins was determined (Table IV). In K-2, who died 43 hours after the isotope was introduced, the highest activity was found in the liver but this had decreased to almost half the value obtained at 7 hours. The specific activity of the muscle was half that of the liver, while the activities of the heart muscle, salivary gland, spleen, lung and pancreas were all approximately of the same order and all about 1/3-1/6 that of

TABLE IV

Incorporation of 2-^{14}C pyruvate into tissue fats and proteins in two cases of decompensated kwashiorkor.

Tissue	Specific activity (cts./min./mg.) of fats K-2	K-1	Tissue	Specific activity (cts./min./mg.) of proteins K-2	K-1
Pancreas	14·5	6·03	Skeletal muscle	24·6	7·5
Salivary gland	6·5	7·1	Thyroid	—	8·9
Skeletal muscle	—	7·6	Salivary gland	9·16	12·6
Thyroid	12·2	8·1	Spleen	9·09	12·1
Lung	12·5	10·4	Lung	11·9	15·8
Liver	10·02	17·4	Liver	56·2(91)*	43·1(126)†
Spleen	15·6	18·9	Pancreas	16·6	—
Kidney	16·7	—	Kidney	3·5	—
Heart muscle	3·1	—	Heart muscle	10·9	—
Rib	6·6	—			

K-1 = 158 hours after isotope.
K-2 = 43 hours after isotope.
† = At 24 hours after isotope.
* = At 7 hours after isotope.

the liver. The lowest radioactivity was found in the kidney. At 158 hours (K-1, Table IV), the specific activity of liver protein had been reduced to one third of what it was at 24 hours and was 3 to 7 times greater than that of the protein of any other tissue examined thus far. The radioactivity of the muscle protein was now 1/3 of what it was in K-2, 115 hours earlier.

With the exception of muscle in K-2 and of liver in both K-1 and K-2, the radioactivity of other tissues at 43 and 158 hours was broadly similar, except for the low kidney activity in K-2. If the total mass of muscle is taken into account in K-2, it follows that the overall radioactivity of the muscles was very much greater than that of the liver. Whether the high activity in the muscles of K-2 can be regarded as a measure of protein synthesis remains an open question. We incline to the view that in the early stages of kwashiorkor, the muscle proteins are being rapidly reduced and these proteins are more dynamically related to the plasma proteins. The specific activity then would represent a measure of the rapidity of exchange between plasma proteins and muscle. During this rapid exchange, the conditions are created for the removal of muscle protein. But, if the high radioactivity in the liver is taken to mean an increased protein synthesis, then by the same token it can be claimed that the muscles in K-2 were also synthesizing plasma proteins, a possibility that still remains to be established.

Our data would show that the muscles and liver are the only two tissues in which the specific activity is significantly elevated in kwashiorkor. However, the other tissues may be implicated in protein metabolism more than is indicated by the specific activity of their respective proteins. This can only be established by careful protein turnover studies.

Specific Activity of the Serum Lipids (*Table V*).

The specific activity of the serum lipids (triglycerides, cholesterol and phospholipids) was determined in 8 cases between 5½ and 7 hours after the administration of the isotope (Table V). With one exception, the concentration of the total serum lipids by gravimetric analysis of the kwashiorkor and control cases ranged from 0·33-0·9%. In the exceptional kwashiorkor case, the serum lipids were over 2 g.%, indicating that the metabolism even of decompensated cases may not be similar (cf. K-1 and K-4) and that there may be a changing pattern of fat metabolism at different stages in the evolution of the syndrome of kwashiorkor.

The specific activity of the unhydrolysed triglycerides was 3-6 times greater in the kwashiorkor cases than in the controls, irrespective of whether the labelled carbon was derived from pyruvate, glucose or acetate (Table V). After hydrolysis of the triglycerides, no activity could be detected in the fatty acids of the kwashiorkor and control cases which received labelled glucose. In all these instances, the specific activity of the unhydrolysed triglycerides could only be attributed to incorporation of the labelled carbon into the glycerol, as previously demonstrated in diabetic baboons (Savage *et al.*, 1960a). The two control cases receiving pyruvate showed a low, but nonetheless significant, amount of radioactivity in the triglyceride fatty acids.

The fact that, after the administration of glucose and pyruvate, radioactivity is found only in the unhydrolysed triglycerides and not in the fatty acids in kwashiorkor is taken to mean either that fatty acid is mobilised from the depots and transported to the liver, and possibly the lung, where it is esterified before release into the circulation as triglyceride or that the fatty acid is synthesised from some other precursor, such as acetate, derived neither from glucose nor from pyruvate.

TABLE V

Specific activity in serum lipids between 5½ and 7 hours after administration of isotope.

Isotope	Case No.	Total Fat (g.%)	Triglycerides (g.%)	Triglycerides: Specific Activity (cts./min./mg.) TGFA‡	Triglycerides: Specific Activity (cts./min./mg.) Unhydrol.	Phospholipids mg.% P	Phospholipids FAPL§ Specific Activity (cts./min./mg.)	Cholesterol (g.%)	Cholesterol Specific Activity (cts./min./mg.)
2-^{14}C Pyruvate	†K-1	0·590	0·139	0	79·7	8·3	17·1	0·148	0
	†K-2	2·015	1·32	0	48·8	16·0	12·2	0·280	0
	C-3	0·467	0·12	2·7	8·9	8·8	4·4	0·148	0
	C-6	0·850	0·46	4·9	8·6	8·1	2·1	0·182	1·0
^{14}C Glucose	†K-4	0·599	0·24	0	32·3	12·5	16·2	0·256	0
	C-5	0·947	0·45	0	16·1	8·2	0	0·290	0
1-^{14}C Acetate	*K-7	0·354	0·098	106	211·0	6·4	100·5	0·096	1604
	C-10	0·335	0·017	37	62·3	7·6	17·3	0·127	67·3

C = Control.
K = Kwashiorkor (*Compensated. †Decompensated).
‡TGFA = glyceride fatty acid.
§FAPL = phospholipid fatty acid.

By contrast, labelled acetate was incorporated to a considerable extent into the fatty acids of the triglycerides both of the control and kwashiorkor cases, being approximately three times as great in the latter as in the former. It is also evident that considerable amounts of the labelled carbon from acetate had been incorporated into the glycerol of the triglycerides, being also 3 times greater in the kwashiorkor than in the control case. Since the kwashiorkor case receiving labelled acetate recovered, it is not possible at this stage to establish whether the significant incorporation of acetate into the triglyceride fatty acid is a feature of the acute episode of decompensated kwashiorkor or whether it is a characteristic of the phase of recovery.

Nevertheless, these observations disclose that acetate is used metabolically in the synthesis of fatty acid of triglyceride by the control as well as by kwashiorkor infants in a manner different from pyruvate and glucose. Pyruvate is therefore not readily converted to active acetate by infants.

The greater specific activity of the fatty acids of the kwashiorkor case (as compared with the control) after the administration of acetate demonstrates that, provided active acetate is available, fatty acid synthesis can proceed at an accelerated rate. Glyceride fatty acid synthesis therefore is preferentially promoted by acetate whereas neither glucose nor pyruvate contribute significantly to glyceride fatty acid synthesis either in the kwashiorkor or in the control infant. Noteworthy is the fact that in infants the glycerol synthesis is more extensively promoted by acetate than by pyruvate or glucose.

Phospholipids (*Table V*)

With the exception of C-5 (the control receiving labelled glucose), (Table V) the phospholipid fatty acids of the kwashiorkor and control cases were labelled to some extent by the carbons derived from glucose, pyruvate and acetate, being 3-6 times greater in kwashiorkor than in the controls. The greatest specific activity was detected in the kwashiorkor infant (K-7) receiving acetate and was 6 times greater than in the control. In the latter (C-10) the specific activity of the phospholipid fatty acids was of a similar order to that of the kwashiorkor cases receiving labelled glucose (K-4) or labelled pyruvate (K-2).

The specific activity of the fatty acids of the triglycerides and of the phospholipids is of a similar order in the kwashiorkor case (K-7) which received acetate, whereas in the other three kwashiorkor cases which received either labelled glucose (K-4) or labelled pyruvate (K-1 and K-2), no activity was detected in the fatty acids of triglycerides but significant activity was detected in the fatty acids of phospholipids. The concentration of the triglycerides or phospholipids in the serum gave no indication of the extent to which the labelled carbon from glucose, pyruvate or acetate would be incorporated into the fatty acids.

Since the method used for separating the fatty acid of phospholipid did not exclude possible contamination with mono- or di-glycerides, we are not able to state with confidence whether the specific activity of the fatty acid of the phospholipid was real or due to a contaminant alone. However, in view of the absence of any radioactivity in the triglyceride fatty acids of K-1, K-2, K-4 and C-5 (Table V), it is unlikely that mono- or di-glyceride fatty acid was responsible for the radioactivity found in the fatty acid of phospholipid in the 3 kwashiorkor cases. It would appear then that the synthesis of the fatty acid of phospholipid in kwashiorkor most likely proceeds in a manner different from that of the fatty acids of triglyceride and that both the carbons of pyruvate and glucose may make some contribution to those of the fatty acids of phospholipids.

Judging from our experiments, acetate is by far the most important precursor of the fatty acid of phospholipid. Whatever the nature of the precursor, the information available demonstrates that the synthesis of fatty acids of phospholipid in kwashiorkor is occurring much more rapidly from pyruvate, glucose and particularly from acetate, than in control cases. This greater incorporation taken in conjunction with the concentration of the serum phospholipids, would suggest a greater turnover rate in kwashiorkor, as compared with the controls.

Cholesterol (*Table V*)

The concentration of serum cholesterol in all the decompensated cases of kwashiorkor was higher than the mean given for kwashiorkor in Cape Town and Pretoria [Schendel and Hansen, 1958; Smit and Pretorius, 1956] and elsewhere including Uganda [Dean and Schwartz, 1953] (see Schendel and Hansen, 1958, for review). One of our cases (K-7), with the lowest serum cholesterol in the series, recovered, whereas all the other kwashiorkor cases died. The level of serum cholesterol as mentioned is therefore not a satisfactory prognostic criterion. With the possible exception of C-6 (control), neither the ^{14}C of pyruvate nor of glucose was incorporated into the serum cholesterol of the kwashiorkor or control cases (Table V). Although some activity was detected in the control receiving pyruvate (C-6), it was very low.

By contrast, significant amounts of radioactivity were detected in the cholesterol of the control and kwashiorkor cases receiving labelled acetate, being more than 24 times greater in the kwashiorkor than in the control case and this despite the lower concentration of cholesterol in the serum of the former (Table V). Unlike glucose or pyruvate, acetate is an important precursor of cholesterol and, when present, can be used for the synthesis of cholesterol in the kwashiorkor infant.

The increased incorporation of labelled acetate into cholesterol in kwashiorkor, in the light of the observations of Gould *et al.*, [1955], would imply that whatever other endocrine dyscrasias there may be, depression of thyroid activity is not sufficient to interfere with cholesterol synthesis. Furthermore, the concentration of cholesterol in the blood gives no indication of the rate of synthesis or of that stored. This is apparent from K-7, in which maximum incorporation of acetate occurred although the level of serum cholesterol was only 96 mg.%.

The increased incorporation of labelled acetate into cholesterol in kwashiorkor indicates that there is no defect in sterol synthesis; moreover, when taken in conjunction with a low concentration of serum cholesterol, the accelerated incorporation would suggest an increased utilisation and/or increased catabolism. Since it is generally maintained that the catabolism of cholesterol is closely linked with the formation of bile acids [Kritchevsky, 1958], an increased excretion of bile acids is not unlikely in kwashiorkor and this may contribute to the onset and maintenance of diarrhoea. However, much of the cholesterol synthesised may in fact accumulate in the liver as cholesterol because the concentration of cholesterol in the liver of kwashiorkor may rise to 2% as compared with 0·5% of the control liver. An increased concentration of cholesterol in kwashiorkor livers has been described elsewhere [Sriramachari and Ramalingaswami, 1953]. In fatal cases, some of this cholesterol may also complex with calcium and bile pigment to form intracellular calculi [Gillman and Gillman, 1951].

In the light of these observations, a low concentration of serum cholesterol in adult Africans or, for that matter, in other ethnic groups, calls for a re-examination of the nature of the underlying metabolism of cholesterol. Clearly in some circumstances, one of which may be kwashiorkor, the rate of cholesterol synthesis may indeed be accelerated and the low serum cholesterol concentration in such circumstances

can only be attributed to greater utilization, storage and/or excretion, possibly as bile acids or some other product.

Radioactivity of the Total Fats in the Tissues

A preliminary analysis was undertaken of the radioactivity in the total extractable lipids of some of the tissues of K-1 and K-2. The results are shown in Table IV and are included in this study for comparison with the other data pertaining to the specific activity of the various lipid fractions of the serum. We do not at this stage propose to comment further on these data.

Specific Activity of Blood Glucose (Figs. 2, 3, 4, and Table VI)

Although the main emphasis of this investigation was placed on the incorporation of the various labelled substrates into fats and proteins and their subsequent oxidation, as assessed by the specific activity of the expired CO_2, nevertheless, some data were obtained about the incorporation of labelled acetate and pyruvate into glucose and of the removal of such labelled glucose from the circulation.

The specific activity of the blood glucose, 50 minutes after the intravenous administration of *uniformly-labelled ^{14}C glucose* into a decompensated kwashiorkor case (K-4), was approximately the same as that of the control after 27 minutes (Table VI and Fig. 2). No activity was detected in the blood glucose isolated from the control after 185 minutes, whereas considerable activity was still present in the blood glucose of the kwashiorkor case after 380 minutes. The blood sugar of the latter (K-4) fell to hypoglycaemic levels during the course of the experiment and was associated with muscular twitchings and nystagmus, while the blood sugar of the control infant was higher at 3 hours than at 27 minutes (Fig. 2).

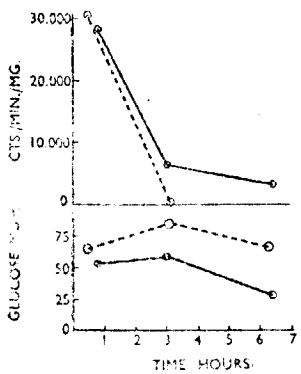


Fig. 2

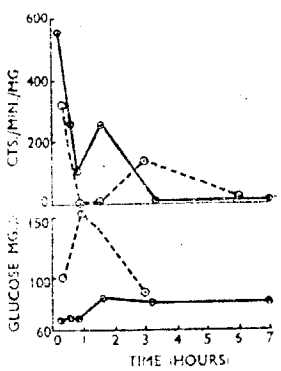


Fig. 3

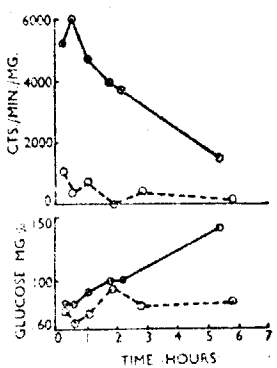


Fig. 4

Fig. 2. Rate of disappearance of radioactivity from the blood glucose after the intravenous administration of uniformly-labelled ^{14}C glucose in kwashiorkor and control cases: interrupted line = control (C-5); solid line = kwashiorkor (K-4).

Fig. 3. Incorporation of 1-^{14}C acetate into blood glucose of kwashiorkor and control cases, and the subsequent disappearance of the synthesized, labelled glucose: interrupted line = control (C-10); solid line = kwashiorkor (K-7).

Fig. 4. Incorporation of 2-^{14}C pyruvate into blood glucose of kwashiorkor and control cases, and the subsequent disappearance of the synthesized, labelled glucose: interrupted line = control (C-8); solid line = kwashiorkor (K-9).

TABLE VI
Incorporation of isotope into blood glucose.

Isotope	Case No.	Time After Isotope Injection (minutes)	Blood Sugar (mg.%)	Specific Activity (cts./min./mg. glucose)
2-^{14}C Pyruvate	*K-9	15	83	5,237
		37	79	5,982
		65	89	4,712
		108	100	3,937
		190	98	3,686
		326	143	1,446
,, ,,	C-8	18	74	1,046
		35	65	398
		65	72	669
		115	94	0
		173	78	354
		345	81	0
,, ,,	C-3	77	130	0
		180	116	0
		440	81	0
,, ,,	C-6	28	61	4,490
		210	109	0
		390	92	0
^{14}C Glucose	‡K-4	50	52	28,100
		180	58	6,256
		380	28	3,350
,, ,,	C-5	27	65	30,600
		185	84	0
		379	66	0
1-^{14}C Acetate	*K-7	14	68	568
		33	70	261
		48	69	105
		95	86	263
		190	82	0
		440	83	0
,, ,,	C-10	20	102	324
		38	126	182
		57	158	0
		90	141	0
		180	89	138
		376		0

C = Control.
K = Kwashiorkor (*Compensated. ‡Decompensated).

In the control (C-10) and in the kwashiorkor case (K-7) receiving 1-^{14}C acetate, relatively little radioactivity was found in the blood glucose. In the control (C-10) there was no radioactivity at the 57th minute whereas it was still present in the kwashiorkor case at 95 minutes (Table VI, Fig. 3). Slight activity in the glucose was detected again in the control at 3 hours. In the absence of food, the level of the blood glucose of the control increased from 102 to 158 mg.% during the 57 minute period

while that of the kwashiorkor case remained relatively unchanged throughout the 6-hour experiment, although the child was fed at the end of the 3rd hour. The increase in the blood sugar of the fasting control infant implies sympathetic stimulation induced during the experiment and also suggests that the liver contained abundant glycogen.

2-^{14}C *Na pyruvate* was incorporated at least 5 times more effectively into the blood glucose of the compensated case of kwashiorkor (K-9) than it was in the control (C-8, Table VI, Fig. 4). In the kwashiorkor (K-9) the blood was not cleared of radioglucose even at the end of 326 minutes, in contrast to the control (C-8) where no activity was detected at 115 minutes after the intravenous administration of the labelled pyruvate. In the control and the kwashiorkor infant the blood sugar did not fluctuate to any marked extent during the experiment.

It can be said that the labelled glucose, whether derived from pyruvate or injected as such, persists for a shorter period in the control than in the decompensated or compensated kwashiorkor case. The rate of removal of labelled glucose and replacement by non-labelled glucose is much more rapid in the control than in the kwashiorkor infant, and is particularly well demonstrated after the intravenous administration of uniformly-labelled ^{14}C glucose.

Utilisation of glucose involves complete oxidation to CO_2 and water, or conversion into glycogen or some other carbohydrate, proteins and fat. Although the specific activity of the expired CO_2 in the control is not greatly different from that in kwashiorkor, nevertheless, it will be shown below that the amount of CO_2 expired in unit time is very much less in kwashiorkor, suggesting a much slower oxidation of glucose. This slower oxidation could contribute to some extent to the persistence of the label in kwashiorkor.

Unfortunately, we were not able to assess the amount of glycogen in the liver after the administration of labelled glucose. If significant amounts of glucose were incorporated into glycogen which, thereafter, was degraded to glucose, then radioactivity should have been detected in the blood glucose of the normal infant at the end of the third hour. Failure to detect radioactivity at that time in the control infant implies either that very little glycogen was labelled by the glucose, or that the glycogen, if labelled, was not contributing significantly to the maintenance of the glucose level in the blood.

It has been commonly asserted that glucose can be derived from the glucogenic amino acids of proteins. As we have demonstrated, some of the ^{14}C of glucose and of pyruvate is consistently incorporated into plasma protein, and into tissue protein (Tables III and IV). While the specific activity of the plasma and tissue proteins is very much less in the control than in the kwashiorkor infant, nevertheless, the overall activity of proteins can be considerable in the control. Since no activity was detected in the blood glucose of the control infant at the end of 3 hours, either the species of plasma and tissue proteins labelled does not normally contribute carbons to the blood glucose, or the isotopic carbons from the plasma and tissue proteins were so heavily diluted by other carbons derived from unlabelled amino acids that they could not be detected by our techniques. On the other hand, the persistence at the end of 6 hours of radioactivity in the glucose of a kwashiorkor case (K-4) receiving ^{14}C glucose may be the result, in part, of the greater incorporation of ^{14}C into the plasma and muscle proteins.

The progressive reduction in the blood sugar to hypoglycaemic levels in K-4 coupled with a persistence of radioactivity in the blood glucose, even to the 6th hour, can hardly be ascribed only to depressed oxidation. In this case, either the liver was

greatly depleted of glycogen or the synthesis of glucose from protein was occurring at a slower rate than the removal of glucose from the circulation, a phenomenon which is an exaggeration of that found in normal, fasted infants. The blood sugar may fall to low levels during food deprivation in young baboons [Gillman *et al.*, 1959] and in human infants [Salomonsen, 1930]. Moreover, we have demonstrated that hypoglycaemia develops rapidly in fasted young rats previously fed a high protein, carbohydrate-free, diet (unpublished data). When liver glycogen is low, the infant, during short periods of food deprivation, cannot replace glucose quickly enough from tissue protein to maintain the blood sugar within normal levels. Moreover, the fact that even in kwashiorkor, metabolites such as acetate and pyruvate are more readily incorporated into proteins than in normal infants, indicates that in the competition between glucose and protein synthesis, the latter may predominate in the face of a falling blood sugar. In these circumstances, the importance of the inclusion of adequate amounts of carbohydrate in the diet of the infant is emphasized.

Since, as we have shown, some glucose can be synthesized from acetate (Table VI), it is not unlikely that the maintenance of the blood sugar of normal infants during short periods of food deprivation is facilitated in part by active acetate made available during fat catabolism. However, judging by the amount of blood glucose synthesized from acetate, it is unlikely that fat contributes more than a very small quota to the maintenance of the resting blood sugar. Whether the sugar formed from acetate represents a net synthesis of carbohydrate remains an open question, although Weinman *et al.*, [1957] suggested that net synthesis of carbohydrate is possible only if there is an influx of non-carbohydrate compounds into the Krebs cycle.

In the normal and in some kwashiorkor cases the maintenance of the blood sugar within normal levels during short fasts is presumably dependent in the first instance upon adequate amounts of glycogen and thereafter on the synthesis of glucose from amino acids. Where the liver contains limited amounts of glycogen, the rate of formation of sugar from amino acids may not keep pace with removal, thus causing a significant decrease in the level of the blood sugar during short fasts. The rapidity of gluconeogenesis from amino acids is in part a function of adreno-cortical activity. In those kwashiorkor cases where the blood sugar continues to fall during a short fast, therefore, a measure of adreno-cortical insufficiency is to be suspected.

Radioactivity of Expired CO_2 (Table VII, Figs. 5, 6, 7)

(1) *CO_2 derived from pyruvate.* From Table VII and Fig. 5 it can be seen that the specific activity of the CO_2 in the one kwashiorkor and two control cases reached a peak somewhere between 5 and 30 minutes while, in K-1, the peak was delayed and probably occurred between the 38th and 77th minute. After the peak was reached, the specific activity declined very rapidly in the control cases (C-3 and C-6) and by the third hour, was 575 and 819 cts./min./mg. CO_2 respectively. In both kwashiorkor cases, the specific activity declined more slowly and by the third hour was still approximately 2,000 cts./min./mg. CO_2. Even at the 7th hour, the specific activity was of an order similar to that attained by the controls at the third hour. At 24 hours, the specific activity was 7-10 times greater in the two kwashiorkor cases than in the control (C-3).

Although there was some loss of CO_2 during collection, nevertheless the data we obtained more than suggest that the total CO_2 formed per minute by the controls was at least 3-4 times greater than in the two decompensated kwashiorkor cases (Table VII).

TABLE VII
Incorporation of isotope into expired CO_2

Isotope	Case No.	Time After Injection of Isotope (minutes)	Specific Activity (cts./min./mg. CO_2) A	CO_2 expired/min. (mg.) B	Overall Activity A × B
2-^{14}C Pyruvate	†K-1	7	1,773	7·1 (11·0)‡	19,503
		38	3,667	11·5	42,193
		77	3,836	10·6	40,661
		127	2,939	10·9	32,035
		194	2,180	11·1	24,198
		315	1,133	4·1 (11·0)‡	12,463
		420	794	10·0	7,940
		24 hrs.	79	12·2	963
,,	†K-2	6	1,749	9·7 (12·2)‡	21,337
		30	3,578	13·6	48,660
		93	2,563	6·0 (12·2)‡	31,268
		159	1,903	9·7 (12·2)‡	23,216
		300	1,094	6·1 (12·2)‡	13,346
		420	710	11·8	8,378
		24 hrs.	117	12·0	1,404
,,	C-3	8	4,153	33·4	138,710
		30	4,648	24·2 (31·5)‡	146,412
		60	3,123	34·6	108,055
		121	1,318	27·5	36,245
		180	575	15·7 (31·5)‡	18,112
		300	139	33·7	4,684
		420	98	29·3	2,871
		24 hrs.	12	30·5	366
,,	C-6	5	2,502	43·9	109,800
		30	3,372	37·9	127,100
		65	2,335	42·2	98,420
		120	1,414	36·7	51,940
		180	819	30·8	25,230
		360	108	40·8	4,422
^{14}C Glucose	†K-4	5	936	16·9	15,820
		30	2,436	19·0	46,290
		60	2,761	23·1	63,750
		120	2,093	14·7	30,760
		200	1,065	17·0	27,280
		395	879	16·4	14,420
		24 hrs.	72·5	21·4	1,552
,,	C-5	5	1,208	34·6	41,790
		30	2,571	45·2	116,200
		60	2,514	36·0	90,510
		120	1,577	45·0	70,980
		180	735	47·5	34,900
		360	115	49·9	5,727
		24 hrs.	5·5	48·8	268

(*Table continued overleaf*)

TABLE VII (*Continued from previous page*)

Isotope	Case No.	Time After Injection of Isotope (minutes)	Specific Activity (cts./min./mg. CO_2) A	CO_2 expired/min. (mg.) B	Overall Activity A × B
1-^{14}C Acetate	*K-7	5	4,311	—	—
		22	3,352		—
		38	2,732		—
		53	2,241		—
		99	1,607		—
		134	1,220		—
		189	824		—
		408	248		—
,,	C-10	5	4,716	49·2	231,084
		23	3,717	56·6	211,869
		47	1,845	38·7	71,955
		60	1,536	44·4	68,198
		97	959	30·2	28,770
		185	368	35·6	13,616
		371	71	32·2	2,272
		403	56	31·0	1,736

C = Control.
K = Kwashiokor (*Compensated. †Decompensated).
‡ = Corrected.

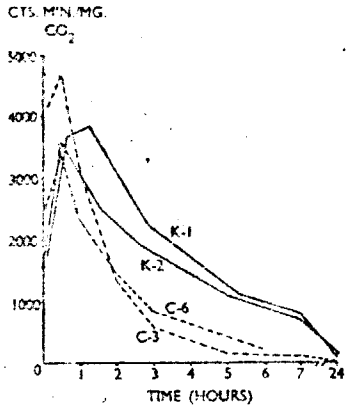


Fig. 5

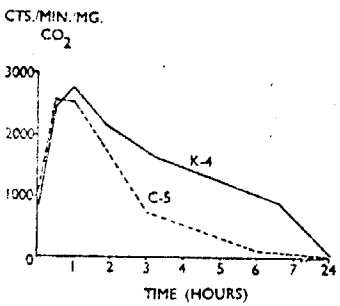


Fig. 6

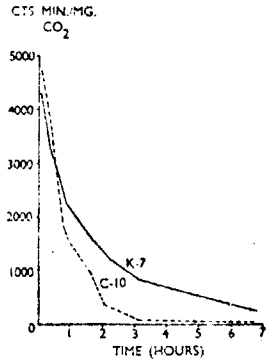


Fig. 7

Fig. 5. Specific activity per mg. CO_2 in expired air formed from the oxidation of 2-^{14}C pyruvate in kwashiorkor and control cases: interrupted line = control; solid line = kwashiorkor.

Fig. 6. Specific activity per mg. CO_2 in expired air formed from the oxidation of uniformly-labelled ^{14}C glucose in kwashiorkor and control cases: interrupted line = control (C-5); solid line = kwashiorkor (K-4).

Fig. 7. Specific activity per mg. CO_2 in expired air formed from the oxidation of 1-^{14}C acetate in kwashiorkor and control cases: interrupted line = control (C-10); solid line = kwashiorkor (K-7).

(2) *CO_2 derived from glucose* (*Fig.* 6, *Table VII*). The specific activity of the CO_2 in the expired air, both in the kwashiorkor and in the control cases, most probably reached a peak between 30 and 60 minutes and thereafter decreased at a much slower

rate in the kwashiorkor infant (K-4) than in that in the control (C-5) (Fig. 6, Table VII). At the third hour, the specific activity of the CO_2 of the decompensated kwashiorkor was at least double and at 24 hours, although further decreased, was approximately 13 times that of the control. The total CO_2 expired/minute by the control was at least 1½-2 times greater than that of the kwashiorkor case. After peak activity is reached, the slope of the declining part of the curve after labelled glucose, as after labelled pyruvate, is therefore much more gradual in the kwashiorkor case than in the control (Fig. 6).

(3) *CO_2 derived from acetate.* (*Fig. 7, Table VII*). The peak of specific activity of the CO_2 in the expired air, both in the kwashiorkor case and in the control was probably reached within the first five minutes. This was followed by a rapid decline in both during the next 50 minutes, but in the control there was already an indication between the 47th and 99th minute that the specific activity of the CO_2 was declining more rapidly than in the kwashiorkor case. At the 3rd hour, the specific activity of the CO_2 was twice as high in the kwashiorkor case as in the control and 4 times greater at approximately 7 hours. It is clear that the peak of CO_2 activity after acetate is attained more rapidly in kwashiorkor as well as in the control than it is after glucose or pyruvate, but that the decay of the curve is much more gradual in the kwashiorkor cases than in the controls, irrespective of the nature of the substrate oxidised. It is to be emphasised, however, that even with the relatively crude methods used, the amount of CO_2 formed in unit time by the controls was found to be at least 1½-4 times greater than that formed by the kwashiorkor cases. The marked reduction in the total amount of expired CO_2 per unit time, indicative of reduced rate of oxidation, may be of considerable importance in determining the rate of removal of glucose from the blood of cases of kwashiorkor.

Apart from the smaller amount of CO_2 expired per unit time, the delayed appearance of the peak of maximum specific activity and slower decay of activity of CO_2, particularly after pyruvate and glucose, suggests that the kwashiorkor case oxidizes ^{14}C uniformly-labelled glucose, 2-^{14}C pyruvate and 1-^{14}C acetate in a manner not appreciably different from the control. The rapid oxidation of 1-^{14}C acetate has been reported in rats as well as in human subjects. In rats, Gould *et al.* [1949] found that after 1-^{14}C sodium acetate, the CO_2 reached a maximum between 10 and 20 minutes as compared with 30 minutes in rats receiving $NaOOC{\cdot}CH_2{\cdot}CH_2{\cdot}^{14}COONa$, both compounds being administered intraperitoneally. The peak of $^{14}CO_2$ after 1-^{14}C sodium acetate administration was missed in man by Shreeve *et al.* [1959] 20 minutes after the administration of the isotope. Harmon *et al.* [1959] remark that "acetate to CO_2 metabolism is apparently one of the most unchangeable processes in the animal biological system. Neither severe fasting nor the final stages of cancer could appreciably change the acetate respiration patterns. As long as the animal remained alive the fraction of the pool oxidised to CO_2 and the rate of oxidation of this fragment remained relatively constant". Even when gravely ill, the kwashiorkor infant is also able to oxidize acetate to CO_2 efficiently.

DISCUSSION

Protein Metabolism

If labelling of proteins, whether derived from amino acids or other precursors, implies synthesis, as Garrow [1959] and Wool [1960] have suggested, then it would

follow that one or possibly more of the individual plasma proteins are being synthesized in kwashiorkor at a rate significantly greater than in control infants. However, caution has been repeatedly advocated in interpreting incorporation of various labelled atoms into proteins as synonymous with synthesis [Tarver, 1954]. As far as can be gathered from the available literature, there is no known method of deciding unequivocally whether or not incorporation can be equated to synthesis. Even if incorporation *does not* represent protein synthesis, the metabolism of the kwashiorkor infant is still markedly different from that of the control. But if incorporation is a reflection of synthesis, then contrary to current views, at least one and possibly more species of the plasma proteins is being synthesized more actively by the kwashiorkor than by the control infant. Presumably then, not only is plasma protein synthesis not impaired in kwashiorkor, but it may indeed be accelerated.

The low plasma protein concentration in kwashiorkor, in association with what appears to be an increased rate of synthesis, would suggest an increased catabolism at least of one or more of the species of the plasma proteins. Studies with ^{131}I labelled albumin in protein-depleted children in Mexico [Gitlin *et al.*, 1958] and in Jamaica [Garrow and Waterlow, 1959], have given no indication of an accelerated half-life of plasma protein, although the usefulness of ^{131}I albumin as a tracer for the study of endogenous albumin turnover rates has not been unequivocally demonstrated [Berson *et al.*, 1953]. Moreover, Garrow [1959] was unable to show an increased catabolism of albumin in protein-depleted dogs.

The low urinary nitrogen, as well as the low blood urea in kwashiorkor at first would also seem to suggest a decreased rate of protein catabolism. But, the fact that non-nitrogen containing precursors are incorporated into plasma protein indicates that these precursors are first synthesised into amino acids, the nitrogen for this synthesis being derived from the catabolism of proteins, at the expense of urea formation. This would imply that the nitrogen of protein catabolism is recycled instead of being converted into urea, thereby accounting for the low blood urea and low excretion of nitrogen in the urine of infants suffering from florid kwashiorkor.

Despite the obvious limitations of using carbon as a tracer for studying protein utilization rates, nevertheless, it would appear that over a period of ten days, the carbon-labelled plasma proteins of the kwashiorkor infants which had received labelled acetate were more sharply reduced than in the control (Fig. 2). This, taken in conjunction with the more extensive and more rapid incorporation of carbons from labelled glucose, acetate and pyruvate into the plasma proteins of kwashiorkor, could be taken to mean a greatly increased utilization of plasma proteins in kwashiorkor rather than any impairment of the synthetic mechanism.

Relationship of liver and tissue proteins to plasma proteins. The close correspondence between the specific activity in the proteins of the liver and plasma of the kwashiorkor infant not only at the end of 7 hours after the administration of isotope but even after 10 days emphasizes the importance of the liver in protein synthesis, and the dynamic relationship between the proteins of the liver and of the plasma. This is all the more remarkable in view of the marked hepatic steatosis [Gillman and Gillman, 1945] and the depletion of liver protein which has been reported in florid kwashiorkor [Waterlow, 1956]. The decrease in specific activity of liver protein at the end of 10 days parallels very closely that of the plasma proteins, suggesting that the turnover rates of the proteins of the liver and plasma may closely follow one another.

In metabolic disorders induced experimentally, either by protein-deficient diets or by plasmapheresis, there is a steady though unequal loss of protein from the different organs and tissues [Solomon and Tarver, 1952; Kosterlitz, 1947; Campbell,

and Kosterlitz, 1948; Waterlow, 1956]. In kwashiorkor, a considerable loss of protein occurs from the liver and from the muscles [Waterlow and Weisz, 1956] and, judging from the atrophy of the mucous membrane of the gut and salivary glands, protein is also lost from these tissues.

In K-1 that came to autopsy 43 hours after administration of the isotope, with the exception of the liver, the specific activity of the skeletal muscle was higher than that of any other tissue (Table IV). Since the muscle constitutes 30-40% of the weight of the infant, the overall activity (i.e. specific activity × total muscle mass) of the muscle proteins of K-1 is therefore greater than that of any other tissue, including the liver. In this case, then, the great mass of muscle tissue was at least exchanging the carbon labels with those in the plasma proteins, and during such exchange the muscle was plundered of its proteins. In K-2, which came to autopsy at 158 hours after administration of the isotope, the specific activity of the muscle proteins was very much lower than in K-1, being scarcely different from that of the thyroid and salivary glands (Table IV). The specific activity of the liver, however, remained elevated, although it was reduced to 1/3 of what it was at the 7th hour after the beginning of the experiment. Whether the radioactivity of the muscle proteins was much higher at any earlier period during the experiment is unknown, for no muscle biopsy was taken at any stage while the infant was alive. At autopsy the muscles were atrophic. Presumably, in the state of active removal of muscle proteins, the specific activity of the muscle tissue rises and in so doing establishes a close functional relation with the plasma proteins. With the rapidly decreasing proteins in the muscles, the specific activity drops, while that in the liver, although reduced, still remains elevated, as compared with that of the muscle and other tissues. If radioactivity is a measure of the metabolic events in muscle, it would seem that during the evolution of decompensated kwashiorkor, at least muscle proteins are actively removed and contribute in some way to the greater radioactivity of the liver proteins. Later, the radioactivity in muscle decreases and reaches a level similar to that of other grossly atrophied tissues, like the pancreas and the thyroid gland.

The gut and protein assimilation. Atrophy of the mucous membrane of the gastro-intestinal tract does not apparently interfere with the dissimilation and absorption of dietary protein. It has been repeatedly demonstrated, through nitrogen balance studies in kwashiorkor infants, that retention of protein is proportional to the dietary load, within limits, even a few days before death [Holemans and Lambrechts, 1955; Waterlow and Wills, 1960]. These observations can be taken to mean that the enzymes of the gut and pancreas, even though much reduced, can nevertheless cope efficiently with the dietary protein. At the time of death, kwashiorkor infants may still be in positive balance both for nitrogen and phosphorus [Waterlow and Wills, 1960]. Furthermore, studies from Greece have demonstrated that in infants suffering from undernutrition and kwashiorkor, the amino acids in the blood, after a test meal of protein, rise in a manner no different from those observed in healthy infants [Matsaniotis, 1957]. But the fact that up to 40% of kwashiorkor infants in our hospital fail to respond to feeding, presumably despite their capacity to absorb and synthesize the dietary protein into plasma protein, more than suggests that, as Waterlow and Wills [1960] have stated, "death is not caused by irreversible failure of nitrogen utilisation," nor presumably can it be said that the plasma protein synthetic mechanism has failed. The accelerated synthesis of proteins even 2 days before death is obviously one expression of a disordered metabolism which in turn may contribute to the demise of the infant.

The accelerated incorporation of acetate, pyruvate and glucose into the plasma protein of kwashiorkor demonstrates in no uncertain manner the profound interrelationship between carbohydrate, fat and protein metabolism. This relationship, clearly evident also in the control infant, is grossly exaggerated in kwashiorkor.

The sparing action of carbohydrate and fat on protein. It has been known for many years that carbohydrate spares protein, as judged by the amount of nitrogen excreted in the urine, especially during starvation [Munro, 1951]. According to Albanese *et al.* [1947] this sparing action is generally assumed to be achieved (a) by preventing the formation of glucose from protein or amino acids, (b) by supplying the total caloric needs in the form of fuel which is mobile and readily available and (c) by providing chemical groupings which are easily convertible to amino acids.

In kwashiorkor, increased incorporation of glucose into protein could not be explained by the abundance of glucose or pyruvate available in the diet, as feeding or food deprivation during the test did not interfere appreciably with the extent to which either pyruvate or glucose were incorporated into the plasma proteins. Geiger *et al.* [1950] found experimentally in rats that the radioactivity of the body proteins after feeding proteins and labelled glucose was not affected, irrespective of whether the proteins and labelled glucose were fed together or separately. Furthermore, the increased incorporation continued in one kwashiorkor infant even while the concentration of blood sugar was falling steadily in the blood (K-4). Since a similar degree of incorporation into plasma proteins was observed in kwashiorkor infants, the blood sugar of which was maintained during the 6-hour test, the extent of incorporation into plasma proteins is evidently not influenced by the level of the blood sugar in kwashiorkor or even in control infants.

Surprising was the incorporation of acetate into plasma proteins of kwashiorkor to an extent as great as that after glucose or pyruvate. However, Swanson [1951] found that unless given extra fat, force-feeding of rats receiving a restricted amount of food inadequate in proteins greatly aggravates the rate of tissue catabolism. In kwashiorkor, there is obviously some disorder of fat metabolism, as reflected by the frequent occurrence of an intensely fatty liver and yet acetate, in such circumstances, is incorporated into the plasma proteins to an extent at least 4 times greater than in the control.

The explanation of the accelerated incorporation of the labelled carbons of glucose, pyruvate and acetate into plasma proteins of kwashiorkor infants is still awaited and the relationship between glucose and fat and protein metabolism remains enigmatic, as is evident from the extensive review of the literature [Munro, 1951].

Fatty Liver

The fatty liver develops early during the acute episode and within a matter of 4-8 days, the fat may increase from a level of 2-4% to over 50% [Gillman and Gillman, 1951]. It was inferred that the fat in the liver was derived predominantly from that mobilised from the depots and, in part, from precursors available from the diet [Gillman and Gillman, 1951]. The data forthcoming from the tracer experiments reported above reveal that neither glucose nor pyruvate are utilized to any extent in the synthesis of cholesterol or of the fatty acids of triglycerides. Since as much as 400 g. of fat may accumulate in the liver in kwashiorkor within 4-8 days, this fat could scarcely have been derived from carbohydrate in the diet during the acute episode when the infant is often severely anorexic.

Considerable amounts of fat presumably are mobilised from the depots and transported as non-esterified fatty acid. On reaching the liver this fat may follow one of

three pathways. First, it may be esterified and, thereafter, discharged into the circulation as triglycerides, thus accounting for the radioactivity in the glycerol in K-1, K-2 and K-4 (Table V). Secondly, the fat may be degraded to acetate which then undergoes complete oxidation; it should be mentioned here that acetate is metabolised to CO_2 more rapidly than glucose and pyruvate by the kwashiorkor infant, as is evident from the observation that the peak of specific activity of CO_2 is reached within 5 minutes after radioactive acetate, as compared with 8-30 minutes after labelled glucose or pyruvate. Thirdly, some of the fat which is degraded to active acetate obviously mingles with the pool of active acetate, and is then resynthesized into cholesterol and into the fatty acids of phospholipids and triglycerides, as happened in K-7, hence accounting for the radioactivity of the cholesterol and of the fatty acids of the triglycerides and phospholipids. The marked synthesis of labelled acetate into cholesterol may in part be responsible for the increased concentration of cholesterol we have found in the livers of some of our cases and also reported by others [Sriramachari and Ramalingaswami, 1953; Delon, 1951].

The information we have presented here demonstrates that despite the marked accumulation of fat, the liver continues to synthesize cholesterol and plasma proteins at a rate greater than normal, if incorporation is a measure of synthesis. Clearly then, a disorder of fat metabolism still remains as one of the obvious characteristics of kwashiorkor.

Some metabolic disorders in kwashiorkor

From a consideration of the data presented above, it would appear that the metabolism of the kwashiorkor infant diverges noticeably from that of the control as measured by the radioactivity of expired CO_2 and the utilization of 2-^{14}C pyruvate, uniformly-labelled ^{14}C glucose and 1-^{14}C acetate for the synthesis of various lipids and plasma proteins. These divergences in metabolism may be examined seriatim:—

(1). The overall production of CO_2 from acetate, glucose and pyruvate is significantly reduced in kwashiorkor as compared with the control.

(2). Qualitatively, as judged by the specific activity of expired $^{14}CO_2$, the kwashiorkor and the control infants apparently use fuels of similar composition to produce CO_2. It is to be emphasized that the peak production of $^{14}CO_2$ from 1-^{14}C acetate by the kwashiorkor and the control infants occurs rapidly and contrasts with the slower development of the peak of CO_2 production from glucose and pyruvate. Despite the profound disorder of metabolism in kwashiorkor, the oxidation of 1-^{14}C acetate to CO_2 still continues actively, although the total amount of CO_2 expired in unit time is greatly reduced.

(3). Pyruvate is synthesized as readily into glucose in kwashiorkor as it is in the control infant, but the slower rate of removal of the labelled glucose may be the consequence, inter alia, of depressed oxidation.

(4). Pyruvate, glucose and acetate are more readily utilized for synthesis of amino acids of the plasma proteins in kwashiorkor than in the control infant.

(5). Neither glucose nor pyruvate contribute carbons for cholesterol synthesis in the kwashiorkor infant. Even in the control, cholesterol is not readily synthesized from glucose or pyruvate.

(6). The cholesterol synthesized from acetate is more than 20 times greater in kwashiorkor than in the control, indicating that the low concentration of cholesterol in the plasma may not be due so much to impaired synthesis as it may be in part to storage in the liver and/or conversion to bile acids

(7). In kwashiorkor, triglyceride fatty acid is not synthesized either from pyruvate or from glucose, but is synthesized in significant amounts from 1-^{14}C acetate. Control infants synthesized small amounts of glyceride fatty acids from pyruvate and much greater quantities from acetate, but apparently none from glucose.

(8). The kwashiorkor infant synthesizes significantly greater amounts of phospholipid fatty acids from pyruvate than does the control, and also synthesizes some phospholipid fatty acid from glucose, whereas the control does not.

(9). While phospholipid fatty acid synthesis occurs from 1-^{14}C acetate both in kwashiorkor and in the control, it is approximately 6 times greater in kwashiorkor.

(10). Glyceride glycerol is synthesized from 1-^{14}C acetate, 2-^{14}C pyruvate, and ^{14}C glucose both by the kwashiorkor and the control infant, being much greater in the former than in the latter.

With one or two exceptions, the data indicated that the difference in the metabolism of kwashiorkor compared with the control is quantitative rather than qualitative. This is clearly demonstrated by the amount of CO_2 expired in unit time and the synthesis of glycerol, fatty acids and cholesterol (Tables V, VII). However, a slower rate of oxidation and utilization may eventually result in a qualitative change. In kwashiorkor the fatty acids synthesized for phospholipids and triglyceride do not apparently proceed along similar pathways. The administration of pyruvate or glucose leads to incorporation in the fatty acids of the phospholipids but not into glyceride fatty acid.

If acetate were an essential precursor of glyceride fatty acid, then, as seems to be indicated in the experiments with 1-^{14}C-acetate in kwashiorkor, neither pyruvate nor glucose is transformed into acetate during oxidation. On this basis, therefore, the pathway, glucose-pyruvate-acetate is either greatly depressed, blocked or is not of much importance.

In the single control infant where labelled glucose was administered, no incorporation occurred into the fatty acids of triglycerides. The oxidation of glucose to acetate in the normal infant also appears to be insignificant, and is only slightly more so than when pyruvate is the precursor. The glucose-pyruvate-acetate pathway, therefore, does not appear to function efficiently, either in normal or in kwashiorkor infants.

It is also apparent from Table V, that acetate is an efficient precursor of cholesterol, especially in kwashiorkor. The fact that neither glucose nor pyruvate in kwashiorkor and control infants was incorporated with cholesterol again emphasizes that the glucose-pyruvate-acetate pathway is greatly depressed or even inoperative in infants. The acetate-fatty acid-cholesterol pathway is operative in both kwashiorkor and control infants, but is greatly enhanced in kwashiorkor.

Fatty acid and cholesterol synthesis in infants, therefore, appear to be dependent on immediate sources of acetate derived neither from glucose nor from pyruvate. However, the one anomalous feature is the synthesis of phospholipid fatty acid from pyruvate both in kwashiorkor and control infants, but from glucose only in kwashiorkor. Until these fatty acids from phospholipids are isolated and identified in pure form a doubt will remain as to whether the radioactivity identified in these fatty acids was not perhaps due to some contaminant from mono- or di-glyceride. On the other hand, fatty acids from triglycerides were so poorly labelled after pyruvate and glucose that it is more than likely that the fatty acids of the phospholipids and of triglycerides are synthesized in unlike ways.

Noteworthy is the consistent observation that glucose, pyruvate and more especially acetate are transformed into glyceride glycerol extensively in the kwashiorkor infant and to a lesser extent in the control. The suggested pathways for glycerol synthesis from acetate might be via the tricarboxylic acid cycle—malate—pyruvate—3-phospho-glyceraldehyde and the oxidative degradation of glycerol might proceed by a reversal of these reactions.

This proposed scheme also allows for the catabolism of glucose and pyruvate to malate through the tricarboxylic acid cycle, and does *not* demand the intermediate formation of acetate. This may explain the absence or the presence in minute amounts only in the control of radioactivity in the glyceride fatty acid and in cholesterol after the administration of 2-^{14}C pyruvate or of uniformly ^{14}C-labelled glucose. Indeed it may not be unlikely that in infants the oxidation of glucose and pyruvate may proceed preferentially via the "malic acid pathway" involving CO_2 fixation, but no intermediate formation of acetate. Furthermore, the delayed peak of activity in the expired CO_2 after glucose and pyruvate administration (as compared with acetate) might also be attributed to this CO_2 fixation prior to complete oxidation. The importance of the malic acid pathway in permitting the entry of pyruvate into the Krebs cycle depends on the velocity of the reaction and this still needs to be established in man. However pyruvate may be oxidised by some other pathway not involving the Krebs cycle.

So far as the synthesis of glycerol is concerned, it would appear that in kwashiorkor the greater incorporation of the labelled carbons of glucose, pyruvate and acetate into glycerol might be a reflection of an increased turnover of non-esterified fatty acids, which are esterified in the liver and possibly in the lung and other tissues. That is to say, at least some fat, the amount of which has still to be determined, is mobilised from the depots and transported in the blood as non-esterified fatty acid. The non-esterified fatty acids, too, might be rapidly oxidized, furnishing active acetate for synthesis again into glyceride and phospholipid-fatty acid as well as cholesterol, thus explaining the labelling of these products when radioactive acetate was administered. Some of the active acetate so formed is also rapidly oxidized to completion – hence, the early peak of labelled CO_2 in the expired air (Figs. 5, 6, 7).

Another defect in the metabolism of the kwashiorkor infant is the accelerated incorporation of acetate into the glyceride- and phospholipid-fatty acid and into cholesterol, as compared with the control. The enhanced glycerol synthesis in both may be one of the stimuli for an enhanced triglyceride synthesis in the liver and in this respect would resemble the stimulation of glyceride fatty acid synthesis by glycerol in the mammary gland [Popjak, 1952]. In kwashiorkor the excess triglyceride synthesized by the liver is deposited excessively in that organ.

All synthetic and catabolic processes involving acetate-fatty acid in the kwashiorkor are apparently intact, and consequently, there are available, adequate amounts, inter alia, of DPNH, TPNH, coenzyme A, riboflavine, thiamin and pantothenic acid. This indicates that in kwashiorkor the metabolic defect is not primarily B-avitaminosis, thus adding confirmation of our view [Gillman and Gillman, 1951]. In this connection it should be mentioned that Burch *et al.* [1957] found no change in the vitamin B_6, DPNH dehydrogenase, malic dehydrogenase, and transaminase content of the liver of kwashiorkor in Guatemala before and after treatment. In our cases certainly all the cholesterol synthetic processes from acetate are not only intact but are also highly efficient.

There also seems little doubt that the systems for the synthesis of at least some of the non-essential amino acids from glucose and pyruvate are intact, and the machinery for the synthesis of at least one or more of the species of plasma proteins.

In this connection attention should be drawn to the importance of the contribution of acetate to the synthesis of some of the amino acids incorporated into one or more of the plasma proteins. The evidence in support of the increased rate of plasma protein synthesis (at least of one of the species of plasma proteins) in kwashiorkor taken in conjunction with the reduced concentration of the plasma proteins and the loss of proteins from the muscles and liver would more than suggest an increased catabolism of protein.

Nutritional requirements of infants

The early age of onset and the age of maximum incidence of kwashiorkor have been well documented. Trowell *et al.* [1954] collated the literature from various parts of the world and found 86% of cases in a series of 1,141 to be under the age of 3 years. In our own series from Johannesburg, 89% were under the age of 3 years [Gillman and Gillman, 1951]. After the age of 3, the incidence drops sharply and kwashiorkor is rarely encountered in children over 5 years. This peculiar age incidence is obviously related to the problem of weaning. That is to say, at weaning, there is some marked disparity between the substrates required for maintaining metabolism and the composition of the diet fed the infant. The emphasis hitherto placed on the inadequacies of the diet is fully justified, as milk and eggs and other high grade foods are not readily available to underprivileged and impoverished people, amongst the infants of whom kwashiorkor is prevalent. The infants of the upper income groups rarely develop kwashiorkor even though the parents of these groups are often as ignorant of the principles of infant nutrition as are those of the lower income groups.

In recent years, so much importance has been attached to the lack of first class proteins in the diet that kwashiorkor has come to be regarded, erroneously in our opinion, as synonymous with protein malnutrition [Brock and Autret, 1952]. There can be no doubt that first class proteins are an indispensable part of the diet of infants, but rational feeding of infants obviously needs to take into account the other constituents of the diet, including the amount and quality of the fat, carbohydrate and minerals, as well as the calorie content. But the diet also requires to be examined in terms of the metabolic peculiarities of the infant in the first 3 to 5 years of life because, apparently, some physiological change occurs in the child after the age of 3-5 when, despite the continued consumption of a bad diet, kwashiorkor no longer occurs. This relation between metabolism and nutrition in developing children has not received the attention it merits.

During the course of the present investigation, apart from disclosing some of the differences in metabolism of the normal and kwashiorkor infants, we were able to obtain some useful information about the peculiarities of metabolism of the normal infant which may broaden present concepts of infant feeding. At least two features of infant metabolism should be particularly stressed. The first concerns the synthesis of lipids. Our studies reveal that up to the age of 2 years and, judging from the age of incidence of kwashiorkor, it may be even much later, the normal infant cannot readily synthesize cholesterol or the fatty acids of triglycerides from glucose or even pyruvate. The pathway glucose-pyruvate-active acetate, does not operate adequately in human infants and may do so later in life. As we shall indicate below, this inability to synthesize cholesterol and triglycerides from carbohydrates makes it obligatory to include some fat in the diet of the weanling.

In the second place, human infants [Salomonsen, 1930], like young baboons [Gillman *et al.*, 1959] cannot usually withstand short periods of food deprivation without developing hypoglycaemia, which may be severe. Even in one of our cases of kwashiorkor, the blood sugar fell from 52 to 28 mg.% in 3 hours (K-4, Table VI).

Such hypoglycaemia can only be taken to mean that, on the one hand, the liver cannot store sufficient glycogen to withstand short periods of food deprivation (and, moreover, the glycogen as we know from baboon experiments is rapidly and markedly depleted during short periods of starvation), and on the other hand, when the infant is fed at irregular intervals, glucose may not be formed sufficiently rapidly from glucogenic amino acids to maintain the blood sugar within normal limits. In older baboons, i.e. over the age of 3½ years, the blood sugar is maintained even during a 3-day fast, indicating that the mechanism controlling gluconeogenesis has become efficient. The age at which the child can withstand short periods of starvation without becoming hypoglycaemic still remains to be established. This capacity to maintain the blood sugar within normal levels during a short period of starvation, as we have shown, is also under the control of the endocrine glands [Gillman *et al.*, 1959]. The rather sluggish manner in which glucose is formed in infants reveals the need for assimilable carbohydrates rather than proteins to be supplied at regular intervals.

In searching for a rational diet for weanlings, and one which would take into account at least the two above mentioned metabolic peculiarities of infants, we were led to examine the composition of breast milk of man and other mammals in relation to growth, as measured by the time it takes the new-born to double its birthweight. From Table VIII, it would appear, inter alia, that (1) the breast milk of every mammal examined contains some fat, the lowest concentration being found in the horse (and the ass) while in some mammals, like the reindeer, whale and porpoise, the concentration may be as high as 22·5%, 44% and 49% respectively of the total content of milk; (2) with the exception of the horse and the ass, the rapidity of post-natal growth is not only a function of the protein concentration, but also of the concentration of fat and lactose in the milk. By and large, the most rapid growth occurs in the rabbit and rat. In these two rodents, the fat and protein content of breast milk are high and the lactose greatly reduced, as compared with that of man. Human milk contains the lowest amount of proteins, namely 1·6% as compared with 14% in the rabbit, and the largest amount of lactose namely, 7% (Table VIII). The human infant takes approximately 180 days to double its birth weight and the rabbit only 6 days. Noteworthy, too, is the fact that 50% of calories are supplied as fat in human breast milk, while the proteins only contribute 9% of the calories. The lactose content of human milk is higher than that reported for any mammal, the horse and the ass, each with

TABLE VIII

Composition of milk of different species and growth rate.
(Modified from Brody's (1945) Table 21.3.)

Species	Time to double birth weight (days)	Constituents as % of whole milk			Caloric values of constituents as percentages of caloric values of milk		
		Fat	Proteins	Lactose	Fat	Proteins	Lactose
Cow	70	4·4	3·8	4·9	53	20	26
Goat	22	4·1	3·7	4·2	54	22	24
Man	180	3·8	1·6	7·0	50	9	41
Sheep	35	6·2	5·4	4·3	59	23	18
Pig	14	7	6	4·0	64	24	12
Dog	9	8·5	7·5	3·7	63	25	12
Rabbit	6	16	14	2	69	27	4
Rat	6	15	12	3	69	25	6
Guinea pig	7	7	5	2	69	22	9
Horse	60	1·6	2·7	6·1	29	22	49

6%, coming closest to man in this respect. Rapid growth apparently seems to be correlated with a high protein and fat content of milk and a decrease in lactose. Obviously other factors are also operative, such as the quality of the protein and of the fat and the quality and quantity of the minerals. It is known, for example, that the proportions of casein, lactglobulin and lactalbumin are not the same in human and cow's milk. Nevertheless, the new-born human infant unquestionably grows efficiently on human breast milk in which 50% of the calories are provided by fat, 41% by lactose and 9% by proteins.

If the composition of human milk is examined in terms of the infant's metabolism, it becomes obvious that the relative inefficiency of the glucose-pyruvate-acetate pathway is counteracted by the fat present in breast milk. This fat can be rapidly oxidized to CO_2, but also at some stage of this oxidation, active acetate becomes freely available for the synthesis of cholesterol and the fatty acids of triglycerides and of phospholipids. Then too, the high lactose content of human breast milk would overcome the need to synthesize glucose from proteins, a mechanism which we have indicated is also rather sluggish in infants. It is to be recalled that both acetate and glucose are also incorporated into plasma proteins, even of the normal infant, a phenomenon which also requires some explanation. Why lactose should be the sugar of choice in breast milk also needs to be explained in terms of the infant's metabolism. After all, galactose in particular concentrations is not entirely without deleterious effects on fat and carbohydrate metabolism, quite apart from its cataract-promoting properties in young animals (See McCollum, *et al.*, 1939, for review).

The protein requirements of the human infant, as assessed from the protein concentration in human milk, are relatively small but, nonetheless, indispensable. The widely recommended practice of making dried skimmed milk available to infants is a step in the right direction of preventing kwashiorkor, but it is to be mentioned in passing that dried skimmed milk contains more than just protein. On the other hand, the importance of fat in the infant's diet has not received the attention it merits. If an infant previously nurtured at the breast is *suddenly* weaned on to a diet which contains insufficient first class proteins, as well as inadequate fat and carbohydrate which is not readily assimilated, and one which is different from lactose, then the basis for the diseases which affect African weanlings so frequently becomes more obvious. Moreover, if it can be shown, in a larger series of infants than we have examined, that the human infant up to the age of 3 years at least, is not easily able to synthesize triglycerides and cholesterol from carbohydrate, then another facet of the eventual explanation for the high incidence of kwashiorkor in this age group may be forthcoming. After the age of 3, and probably about 5 years, as part of the developmental process and accompanied by a different endocrine configuration, fat synthesis can proceed more easily from carbohydrate, and fat in the concentrations previously available in milk, may no longer become as important as in the earlier years of life. The bottle-neck in fat synthesis is removed and this may also contribute to the sharp decline in the incidence of kwashiorkor in children over the age of 5 years.

While not in any way denying the importance of minimal amounts of first class proteins in the diet of weanlings, attention is now also directed to the importance of dietary fat and of an assimilable carbohydrate. It may be significant that one of the outstanding features of the disorder of metabolism in kwashiorkor is a profound alteration in fat metabolism, reflected in the massive accumulation of lipid in the liver.

SUMMARY

The metabolism of uniformly-labelled ^{14}C glucose, 2-^{14}C pyruvate and 1-^{14}C acetate was investigated in 5 cases of kwashiorkor (2 compensated and 3 decompensated) and 5 control infants. Observations were made on (1) the oxidation of these isotopes to carbon dioxide, as measured in the expired air; (2) their incorporation into proteins, cholesterol, fatty acids of triglycerides and phospholipids, glycerol and glucose of the plasma and/or serum and, where circumstances permitted, into the liver and other tissues; (3) the removal of synthesized, labelled glucose and of administered glucose from the circulation, and (4) the incorporation of the isotopes into the liver and plasma proteins during the first 5-7 hours of the experiment and for 10 days thereafter.

From a consideration of the results obtained, it was shown that the metabolism of the kwashiorkor infant is similar in some respects, and differs in others from that of the control infant. These points are summarised in the discussion (pages 53-54).

From an analysis of these metabolic similarities and differences in the kwashiorkor and the control infant, it was concluded that (1) the pathway glucose-pyruvic acid to active acetate is greatly depressed or even inoperative in infants; (2) the pathway acetate-fatty acid-cholesterol is efficient both in kwashiorkor and control infants, but is enhanced in the former: (3) in infants (kwashiorkor and control) under the age of 3 years, the oxidation of glucose may proceed preferentially via the "malic acid pathway" involving CO_2 fixation, but without the intermediate formation of active acetate; (4) in view of the efficient synthesis of triglycerides, cholesterol and phospholipids from acetate, it would appear that in kwashiorkor there were available adequate amounts, inter alia, of DPNH, TPNH, coenzyme A, riboflavin, thiamin and pantothenic acid. By taking into account the age of maximum incidence of kwashiorkor, together with some of the metabolic peculiarities and the composition of human breast milk, it became apparent that the normal infant (at least until the age of 3 years) cannot easily synthesize fat from carbohydrate, and that gluconeogenesis from non-carbohydrate sources is sluggish. The evidence seemed more than suggestive that the normal infant was dependent not only on dietary protein, but also on adequate amounts of dietary fat and carbohydrate. Finally, it was concluded that the syndrome of kwashiorkor could *not* be ascribed primarily to an insufficiency of first class proteins.

We wish to acknowledge our great indebtedness to Dr. H. Wallace, Head of the Department of Paediatrics, for his encouragement and his deep interest in this study, and to him and to Dr. P. Klenerman for making available to us the clinical cases without which this investigation would not have been possible. Thanks are also due to Professor E. B. Adams, Head of the Department of Medicine, and to Dr. S. Disler, Superintendent of King Edward VIII Hospital. Mr. D. Donly gave valuable assistance with the radioactivity determinations. The authors also wish to acknowledge the excellent technical assistance of Mr. H. Klomfass, Mrs. R. Agulhas, and Mr. L. Notha.

This work was aided in part by a grant from the National Institutes of Health: Grant No. A 3532 (Cl) Nutr. to T.G.

REFERENCES

ALBANESE, A. A., IRBY, V., FRANKESTON, J. E. and LEIN, M. (1947). Effect of carbohydrate feeding on output of urinary amino acid. *Amer. J. Physiol.*, 150, 389-393.

BEHAR, M., VITERI, F., BRESSANI, R., ARROYAVE, G., SQUIBB, R. L. and SCRIMSHAW, N. S. (1958). Principles of treatment and prevention of severe protein malnutrition in children (kwashiorkor). *Ann. N.Y. Acad. Sci.*, 69, 954-968.

BERSON, S. A., YALLOW, R. S., SCHREIBER, S. S. and POST, JOSEPH. (1953). Tracer experiments with ^{131}I-labelled human serum albumin: distribution and degradation studies. *J. clin. Invest.*, 32, 746-768.

BROCK, J. F. and AUTRET, M. (1952). *Kwashiorkor in Africa.* (Food and Agricultural Organisation, Rome). F.A.O. Nutritional Studies No. 8.

BRODY, S. (1945). *Bio-energetics and growth.* (Reinhold Publishing Co., New York).

BURCH, H. B., ARROYAVE, G., SCHWARTZ, R., PADILLA, A. M., BEHAR, M., VITERI, F. and SCRIMSHAW, N. S. (1957). Biochemical changes in the liver associated with kwashiorkor. *Fed. Proc.*, 16, 160.

CAMPBELL, R. M. and KOSTERLITZ, H. W. (1948). The relationship between losses in labile liver cytoplasm and urinary nitrogen excretion. *Biochem. J.*, 43, 416-419.

COLLIS, W. R. F. (1958). Discussion at conference on nutrition. *W. Afr. med. J.*, 7, 37.

DEAN, R. F. A. and SCHWARTZ, R. (1953). Serum chemistry in uncomplicated kwashiorkor. *Brit. J. Nutr.*, 7, 131-147.

DELON, J. (1951). *Maroc. med.*, 30, 578-583. (Cited by Trowell *et al.*, 1954.)

GARROW, J. S. and WATERLOW, J. C. (1959). Observation on Evans Blue dye as a tracer for human plasma albumin. *Clin. Sci.*, 18, 35-43.

GARROW, J. S. (1959). The effect of protein depletion on the distribution of protein synthesis in the dog. *J. clin. Invest.*, 38, 1241-1250.

GEIGER, E., WICK, A. and BANCROFT, R. W. (1950). Proc. 18th Internat. Congr. Physiol. p. 214. (Quoted by Munro, 1951.)

GILBERT, C., GILLMAN, J. and SAVAGE, N. (1960). Persistence of the hyperglycaemia and the reduction in lipaemia and ketonaemia of diabetic baboons following adrenalectomy. *S. Afr. J. med. Sci.*, 25, 77-80.

GILLMAN, J. and GILBERT, C. (1958a). The fatty liver of endocrine origin, with special reference to the fatty liver of malnourished African infants. *Brit. med. J.*, i, 57-63.

GILLMAN, J. and GILBERT, C. (1958b). The endocrine mediation of individuality as assessed from a study of the serum lipids and ketones in depancreatised baboons. *S. Afr. J. med. Sci.*, 3, 35-37.

GILLMAN, J. and GILBERT, C. (1959). The dependence of the fatty liver of depancreatized baboons on adrenocortical activity. *S. Afr. J. med. Sci.*, 24, 150-151.

GILLMAN, J., GILBERT, C. and ALLAN, J. C. (1958a). The relationship of hyperglycaemia to hyperlipaemia and ketonaemia in depancreatized baboons. *J. Endocrinol.*, 17, 349-362.

GILLMAN, J., GILBERT, C., EPSTEIN, E. and ALLAN, J. C. (1958b). Endocrine control of blood sugar, lipaemia and ketonaemia in diabetic baboons. *Brit. med. J.*, ii, 1260-1263.

GILLMAN, J., GILBERT, C., EPSTEIN, E. and ALLAN, J. C. (1958c). Ketogenic action of human anterior pituitary in diabetic baboons. *Lancet*, i, 1,226.

GILLMAN, J., GILBERT, C. and TROUT, G. E. (1957). Preliminary report on dissociation in the behaviour of the components of the serum lipids in depancreatized-hypophysectomized baboons. *S. Afr. J. med. Sci.*, 22, 38-39.

GILLMAN, J., GILBERT, C. and SAVAGE, N. (1959). Serum lipid, blood glucose and liver fat in normal fasting baboons with a consideration of some of the controlling endocrine factors. *S. Afr. J. med. Sci.*, 24, 115-124.

GILLMAN, T. and GILLMAN, J. (1945). Hepatic damage in infantile pellagra and its response to vitamin, liver and dried stomach therapy as determined by repeated liver biopsies. *J. Amer. med. Ass.*, 129, 12-19.

GILLMAN, J. and GILLMAN, T. (1951). *Perspectives in human malnutrition.* (Grune and Stratton, New York.)

GITLIN, D., CRAVIOTO, J., FENK, S., MONTANO, E. L., GALVAN, R. R., GOMEZ, F. and JANEWAY, C. A. (1958). Albumin metabolism in children with protein malnutrition. *J. clin. Invest.*, 37, 682-686.

GOULD, R. G., MAROTT SINEX, F., ROSENBERG, I. N., SOLOMON, A. K. and BAIRD HASTINGS, A. (1949). Excretion of radioactive CO_2 by rats after administration of isotopic bicarbonate, acetate and succinate. *J. biol. Chem.*, 177, 295-301.

GOULD, R. G., LEROY, G. V., OKITA, G. T., KABARA, J. J., KEEGAN, P. and BERGENSTALL, D. M. (1955). The use of ^{14}C-labelled acetate to study cholesterol metabolism in man. *J. Lab. clin. Med.*, 46, 372-384.

HARVON, D. H., KIRK, M. R. and TOLBERT, B. M. (1959). Effect of cancer on oxidation of labelled acetate and glycine to $C^{14}O_2$. *Amer. J. Physiol.*, 196, 265-268.

HOLLMANS, K. and LAMPRECHTS, A. (1955). Nitrogen metabolism and fat absorption in kwashiorkor. *J. Nutrit.*, 56, 477-494.

KOSTERLITZ, H. W. (1947). The effect of changes in dietary protein on the composition and structure of the liver cell. *J. Physiol. (Lond.)*, 106, 194-210.

KRITCHEVSKY, D. (1958). *Cholesterol.* (John Wiley & Sons, New York).

MANCHESTER, K. L. and YOUNG, F. G. (1959). Location of ^{14}C in protein from isolated rat diaphragm incubated in vitro with [^{14}C] amino acids and with $^{14}CO_2$. *Biochem. J.* 72, 136-141.

MATSANIOTIS, N. S. (1957). Studies on protein metabolism in undernourished children. *J. Pediat.*, **51**, 267-284.

McCOLLUM, E. V., ORENT-KEILES, E. and DAY, H. G. (1939). *The newer knowledge of nutrition* (Macmillan Co., New York).

MUNRO, H. N. (1951). Carbohydrate and fat as factors in protein utilization and metabolism. *Physiol. Rev.*, **31**, 449-488.

PORJAK, G. (1952). Fat synthesis from small molecules. *Biochemical Soc. Symposia*, No. 9, p. 37 (Cambridge University Press).

SALOMONSEN, L. (1930). Effects of insulin and epinephrine on blood acetone in fasting children. *Amer. J. Dis. Child.*, **40**, 718-724.

SAVAGE, N. S., GILLMAN, J. and GILBERT, C. (1960). Influence of insulin on the incorporation of 2-C^{14} sodium pyruvate into glyceride glycerol in diabetic and normal baboons. *Nature*, **185**, 168-169.

SAVAGE, N., GILLMAN, J. and GILBERT, C. (1960a). Unimpaired synthesis of fatty acids and altered synthesis of glycerol to triglycerides in diabetic baboons. *S. Afr. J. med. Sci.*, **25**, 19-32.

SAVAGE, N., GILLMAN, J. and GILBERT, C. (1960b). Increased synthesis of phospholipid fatty acid from 2-^{14}C pyruvate in the diabetic baboon. *S. Afr. J. med. Sci.*, **25**, 81-87.

SAVAGE, N., GILLMAN, J. and GILBERT, C. (1960c). Unimpaired synthesis of cholesterol from 2-^{14}C pyruvate in diabetic baboons. *S. Afr. J. med. Sci.*, **25**, 71-75.

SCHENDEL, H. E. and HANSEN, J. D. L. (1958). Studies on fat metabolism in kwashiorkor. *Metabolism*, **7**, 731-741.

SCRIMSHAW, N. S., BEHAR, M., ARROYAVE, G., VITERI, F. and TEJADA, C. (1956). Characteristics of kwashiorkor (sindrome pluricarenciol de la infancia). *Fed. Proc.*, **15**, 977-985.

SHREEVE, W. W., HENNES, A. R. and SCHWARTZ, R. (1959). Production of $C^{14}O_2$ from 1 and 2-C^{14} acetate by human subjects. *Metabolism*, **8**, 741-756.

SMIT, Z. M. and PRETORIUS, P. J. (1957). Some biochemical changes in kwashiorkor. *S. Afr. J. lab. clin. Med.*, **3**, 142-148.

SOLOMON, G. and TERVER, H. (1952). The effect of diet on the rate of loss of labelled amino acid from tissue proteins. *J. biol. Chem.*, **195**, 447-452.

SOMOGYI, M. (1945). Determination of blood sugar. *J. biol. Chem.*, **160**, 69-73.

SRIRAMACHARI, S. and RAMALINGASWAMI, V. (1953). *Indian J. Pediat.*, **20**, 1-6. (Quoted by Trowell *et al.*, 1954).

SWANSON, P. R. (1951). Influence of non-protein calories on protein metabolism. *Fed. Proc.*, **10**, 660-669.

TARVER, H. (1954). Peptide and protein synthesis: in *The Proteins*, **2**, part B, p. 1,199. Eds. Neurath and Bailey. (Academic Press, New York).

TROWELL, H. C., DAVIES, J. N. P. and DEAN, R. F. A. (1954). *Kwashiorkor*. (Edward Arnold, London).

VAN SLYKE, D. D., PLAZIN, J. and WEISIGER, J. R. (1951). Reagents for the Van Slyke-Folch wet carbon combustion. *J. biol. Chem.*, **191**, 299-304.

WATERLOW, J. C. (1956). The protein content of liver and muscle as a measure of protein deficiency in human subjects. *W. Ind. med. J.*, **5**, 167-174.

WATERLOW, J. C. (1957). Observations on enzymes in human liver. *Acta Un. int. Cancr.*, **13**, 704-711.

WATERLOW, J. C. (1958). Observations on enzymes in the human liver, general considerations and preliminary results. *W. Ind. med. J.*, **7**, 44-52.

WATERLOW, J. C. and WEISZ, T. (1956). Fat, protein and nucleic acid content of liver in malnourished human infants. *J. clin. Invest.*, **35**, 346-354.

WATERLOW, J. C. and WILLS, V. (1960). Balance studies in malnourished Jamaican infants. *Brit. J. Nutr.*, **14**, 183-198.

WEINMAN, E. O., STRISOWER, E. H. and CHAIKOFF, I. L. (1957). Conversion of fatty acids to carbohydrate: application of isotopes to this problem and role of the Krebs cycle as a synthetic pathway. *Physiol. Rev.*, **37**, 252-272.

WOOL, I. G. (1960). Stimulation of ^{14}C histidine incorporation into protein of isolated diaphragms by non-utilizable sugars. *Amer. J. Physiol.*, **198**, 57-59.

ZILVERSMIT, D. B., CHAIKOFF, I. L., FELLER, D. D. and MASORO, E. J. (1948). Oxidation of glucose-labelled with radioactive carbon by normal and alloxan-diabetic rats. *J. biol. Chem.*, **176**, 389-400.

Proceedings

of the

Society

for

Experimental Biology and Medicine

Proc. Soc. Exp. Biol. Med. 130 (4), 1033-41, 1969

Vol. 130 April, 1969 No. 4

Effects of Monosaccharides, Acetate, Butyrate, Lactate, and Pyruvate on the Developed Tension of Isolated Rat Atria[a] (33744)

A. L. Greco,[1] J. L. Lacuara,[1] M. F. Greco,[1] and E. A. Sayeso

Segunda Cátedra de Fisiología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina, and Department of Pharmacology, University of Southern California School of Medicine, Los Angeles, California 90033

In previous investigations we studied the importance of glucose for the contraction of isolated rat atria (1, 2). We found that the removal of glucose from the medium as well as the addition of 2-deoxyglucose (2-DG) resulted in a rapid decrease in the isometric developed tension and ATP levels of rat atria. The addition of pyruvate or lactate to this hypodynamic preparation restored ATP levels but failed to completely restore the contractile tension to the control levels (1, 2). Furthermore, we have also demonstrated that high concentrations of pyruvate depressed the developed tension of atria suspended in glucose medium. The depression occurred even when the entry of pyruvate into the tricarboxylate cycle was blocked by arsenite (2) or when the conversion of pyruvate to lactate was inhibited by oxamate (3). Inasmuch as high concentrations of pyruvate can inhibit the uptake of glucose by the heart as well as several steps of the glycolitic pathway (1-7) we suggested that such metabolic influences could possibly explain the negative inotropic effect observed after pyruvate.

All the above evidence indicated that glucose plays a definite role in the maintenance of atrial developed tension. The present study was undertaken to determine [illegible] rather well [illegible] and [illegible] the [illegible] in the [illegible] isolated rat atria [illegible] the [illegible] of [illegible], acetate, and pyruvate [illegible] found that [illegible] could [illegible]

[a] Supported by Grants [illegible], GB-4127 and 14114 from the National Institute of Health, the Fund Science Research Council, Consejo Nacional de Investigaciones Científicas, Tecnicas de la República Argentina, respectively.

[1] [illegible] Fellows from the [illegible]

TABLE I. Initial Developed Tension Magnitude and Stability of the Developed Tension of Isolated Rat Atria in Glucose, Mannose, Fructose, or 3-*O*-Methylglucose.[a]

Substrate	Conc (mM)	Initial developed tension magnitude (mg)	Stability of the developed tension (% change) with time				
			10 min	30 min	60 min	90 min	120 min
Glucose	5.5	438 ± 28.9 (22)	−1.2 ± 0.7 (14)	−2.8 ± 0.9 (14)	−6.3 ± 1.1 (12)	−6.2 ± 1.7 (8)	−7.0 ± 1.8 (6)
	11.0	471 ± 30.7 (20)	0.0 ± 0.1 (6)	−0.3 ± 0.1 (6)	−1.9 ± 0.2 (6)	0.0 ± 0.1 (6)	+0.5 ± 0.03 (4)
Mannose	5.5	521 ± 38.2 (8)	−0.8 ± 0.06 (8)	−1.8 ± 1.6 (8)	−5.8 ± 1.9 (8)	−6.9 ± 1.0 (8)	−8.6 ± 1.5 (4)
	11.0	453 ± 32.1 (6)	−3.1 ± 1.3 (6)	−5.5 ± 1.4 (6)	−10.9 ± 1.7 (6)	—	—
Fructose	5.5	304 ± 37.1 (6)	−4.0 ± 1.3 (6)	−11.1 ± 2.2 (6)	−21.5 ± 3.0 (6)	—	—
	11.0	287 ± 26.1 (6)	−8.7 ± 1.8 (4)	−16.4 ± 1.5 (4)	−25.2 ± 3.2 (5)	—	—
3-*O* Methylglucose	5.5	186 ± 18.7 (4)	−8.7 ± 0.8 (4)	−23.5 ± 2.0 (4)	−30.4 ± 2.9 (4)	—	—
	11.0	150 ± 20.6 (4)	−13.3 ± 1.5 (4)	−34.2 ± 0.6 (4)	−46.0 ± 2.5 (4)	—	—
None	—	196 ± 34.8 (9)	−7.5 ± 1.3 (5)	−22.0 ± 2.8 (5)	−40.7 ± 3.6 (5)	—	—

[a] Stability of the developed tension with time is expressed as percentage of change from the initial control values. In all cases the values are mean ± SEM; the number of atria given in parentheses.

stimulate atrial developed tension in a sustained form, glucose being the most effective. Furthermore, increasing concentrations of 3-*O*-methylglucose, acetate, butyrate, and pyruvate depressed the developed tension magnitude of isolated rat atria.

Methods. Atria from decapitated male rats were suspended in a tissue chamber filled with a modified Krebs Ringer bicarbonate solution (KRB) composed as follows (mM): Na^+, 145; K^+, 6.0; Ca^{2+}, 1.6; Mg^{2+}, 1.33; Cl^-, 126; CO_3^{2-}, 1.33; HCO_3^-, 25.0; and PO_4^{3-}, 1.20. The substrate for this solution was glucose, mannose, methyl-3-*O*-methylglucose, lactate, acetate, butyrate, or pyruvate, at 5.5 or 11.0 mM. The solution was oxygenated with a mixture of 95% O_2, 5% CO_2 (at a flow rate of 150 ml/min) and maintained at pH 7.4 at 30°C. [illegible] Application. The average lactate concentration found in 10 mannose solutions incubated during 60 min is 1.43 [illegible] from with 5.5 mM glucose was 2.17 [illegible] (w/w) (mean ± SEM). A constant resting tension of 750 mg was applied to the atria by means of a micrometer device. The isometric atrial developed tension was recorded with the aid of a strain gauge connected to a direct writing oscillograph. Atria were stimulated with a pulse voltage of 5.7 V and 1 msec duration at a constant rate of 200 min. The equilibration period was 60 min. The recorded tension at the end of the equilibration period is referred to the [illegible] the initial developed tension. The effects of increasing concentrations of the [illegible] resulting from the addition of substrates were compared with the [illegible] the data [illegible] [illegible]

TABLE II. Initial Developed Tension Magnitude and Stability of Developed Tension of Isolated Rat Atria in Pyruvate, Lactate, Acetate, or Butyrate.[a]

Substrate	Conc (mM)	Initial developed tension magnitude (mg)	Stability of developed tension (% change) with time			
			10 min	30 min	60 min	90 min
Pyruvate	5.5	451 ± 28.0 (16)	−0.4 ± 0.08 (5)	−2.8 ± 0.5 (5)	−3.7 ± 0.6 (5)	−6.5 ± 1.5 (5)
	11.0	460 ± 44.9 (9)	−0.3 ± 0.1 (7)	−1.3 ± 0.2 (7)	−3.9 ± 0.3 (4)	−5.1 ± 0.9 (4)
Lactate	5.5	415 ± 29.6 (8)	−0.6 ± 0.5 (6)	−1.7 ± 1.0 (6)	−4.8 ± 1.0 (6)	−6.1 ± 1.3 (6)
	11.0	433 ± 33.2 (7)	−1.1 ± 0.4 (5)	−2.2 ± 0.8 (5)	−4.0 ± 1.1 (5)	−5.8 ± 1.4 (5)
Acetate	5.5	455 ± 50.3 (8)	+0.1 ± 0.2 (8)	−2.2 ± 1.8 (8)	−4.4 ± 3.6 (8)	−9.1 ± 4.3 (8)
	11.0	413 ± 41.5 (6)	−0.2 ± 0.2 (6)	−3.6 ± 2.1 (6)	−5.1 ± 1.6 (6)	−9.9 ± 1.0 (6)
Butyrate	5.5	403 ± 22.5 (8)	−0.5 ± 0.4 (8)	−2.2 ± 1.2 (8)	−4.6 ± 2.2 (8)	−10.1 ± 3.5 (8)
	11.0	498 ± 37.6 (7)	−0.7 ± 0.6 (7)	−0.2 ± 0.5 (7)	−4.7 ± 3.2 (7)	−5.6 ± 4.1 (7)

[a] Stability of the developed tension with time is expressed as percentage of change from the initial control values. In all cases the figures are mean ± SEM; number of atria given in parentheses.

tration upon adding the sodium salts of the substrates were determined with equivalent additions of NaCl (2). The addition of substrates did not produce significant changes in the pH of the medium (>0.1 pH unit). Mean values of developed tension under different experimental conditions were compared using Student's t test. Differences were considered significant if $p \leq 0.05$ or less.

Results. Initial developed tension magnitude and stability of developed tension of isolated rat atria suspended in different substrates. Rat atria were set up in a medium containing glucose, mannose, fructose, 3-O-methylglucose, lactate, acetate, butyrate, or pyruvate as the substrate; the initial developed tension, immediately after the 60 min of equilibration period, was determined (Tables I and II). It is evident that when glucose, mannose, acetate, lactate, butyrate, or pyruvate were present the developed tension was significantly greater than when fructose or 3-O-methylglucose was the substrate. On the other hand, atrial developed tension in fructose was significantly greater than in 3-O-methylglucose or in the substrate-free condition. Increasing the substrate concentration from 5.5 to 11.0 mM had no significant effect on the magnitude of the initial developed tension.

Tables I and II also shows the effects of substrates on the stability of the developed tension magnitude with time. It is evident that the stability was influenced not only by the substrates, but also with glucose by the concentration. Comparison of the stability at 5.5 and 11.0 mM glucose shows that the decrement with time was significantly less when the concentration of this substrate was 11.0 mM. On the other hand, increasing concentrations of mannose, 3-O-methylglucose, fructose, acetate, lactate, butyrate, or pyruvate from 5.5 to 11.0 mM did not prevent the decrement of developed tension with time. Although fructose was significantly better than 3-O-methylglucose or the substrate-free control, it was not as effective as glucose, mannose, lactate, acetate, butyrate, or py-

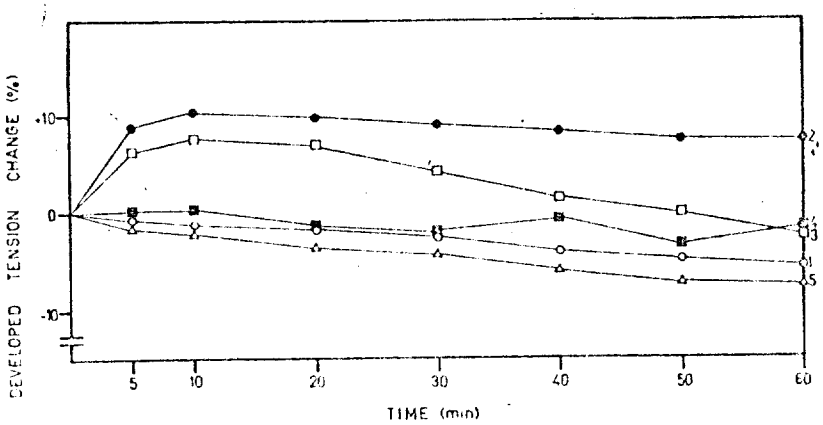


FIG. 1. Effects of 5.5 m*M* glucose, mannose, fructose or 3-*O*-methylglucose on the developed tension of atria suspended in glucose medium. Atria were equilibrated in 5.5 m*M* glucose and the initial developed tension was recorded; 5.5 m*M* of the different substrates were added immediately after (0 time). Each point represents the mean of 5–14 different experiments: Curve 1, controls in 5.5 m*M* glucose with no additions (14 atria); curve 2, glucose (13 atria); curve 3, mannose (7 atria); curve 4, fructose (5 atria); curve 5, 3-*O*-methylglucose (6 atria).

ruvate in maintaining the magnitude of atrial developed tension.

The effects of glucose, mannose, fructose, 3-O-methylglucose, pyruvate, acetate, and butyrate on the developed tension of isolated rat atria suspended in Krebs–Ringer–bicarbonate–glucose medium. The ability of several substrates to modify the isometric contractile tension of isolated rat atria suspended in KRB medium containing 5.5 m*M* glucose

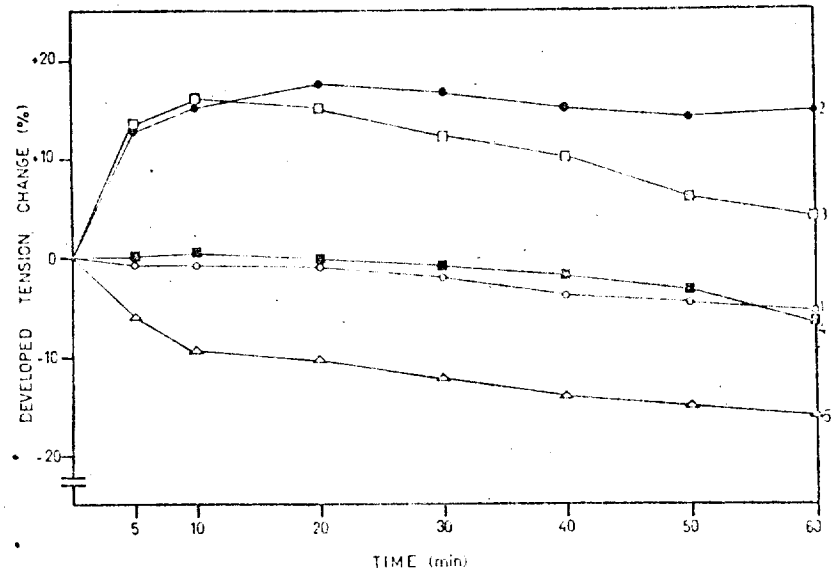


FIG. 2. Effects of 11.0 m*M* glucose, mannose, fructose or 3-*O*-methylglucose on the developed tension of atria suspended in glucose medium. Conditions and details as described in Fig. 1. Curve 1, controls in 5.5 m*M* glucose with no additions (14 atria); curve 2, glucose (7 atria); curve 3, mannose (5 atria); curve 4, fructose (5 atria); curve 5, 3-*O*-methylglucose (5 atria).

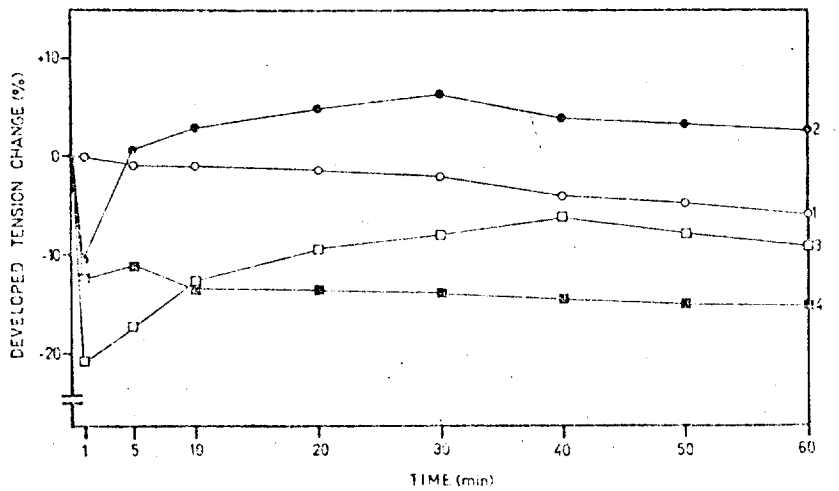


FIG. 3. Effects of 2.75 m*M* pyruvate, acetate or butyrate on the developed tension of atria suspended in glucose medium. Conditions and details as described in Fig. 1. Curve 1, controls in 5.5 m*M* glucose with no additions (14 atria); curve 2, pyruvate (7 atria); curve 3, acetate (8 atria); and curve 4, butyrate (8 atria).

was investigated (Figs. 1–5). As shown in the previous section the developed tension of atria suspended in KRB medium with 5.5 m*M* glucose, exhibited a slight and progressive decrement with time. The addition at 0 time of 5.5 or 11.0 mM glucose, to make a final concentration in the medium of 11.0 or 16.5 m*M*, respectively, resulted in a sustained positive inotropic effect (Figs. 1 and 2, curves 2). Although 5.5 or 11.0 m*M* mannose also stimulated the atria (Figs. 1 and 2, curve 3) the effect was less sustained than with identical concentrations of glucose; this being particularly evident 40 min after the addition of mannose (at the time when the changes of atrial contractile tension were significantly smaller than those after the addition of glucose). On the other hand 5.5 or 11.0 m*M* fructose (Figs. 1 and 2, curve 4) as well as 5.5 m*M* 3-*O*-methylglucose (Fig. 1,

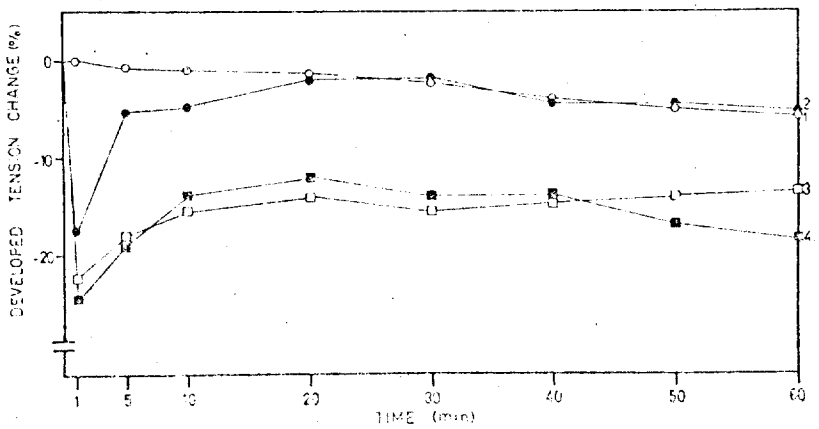


FIG. 4. Effects of 5.5 m*M* pyruvate, acetate or butyrate on the developed tension of atria suspended in glucose medium. Conditions and details as described in Fig. 1. Curve 1, controls in 5.5 m*M* glucose with no additions (14 atria); curve 2, pyruvate (12 atria); curve 3, acetate (12 atria); curve 4, butyrate (9 atria).

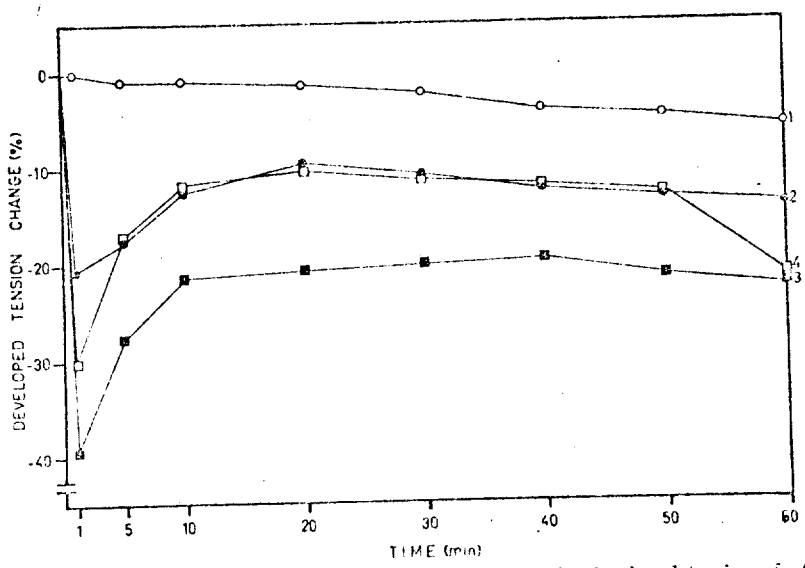


FIG. 5. Effects of 11.0 m*M* pyruvate, acetate or butyrate on the developed tension of atria suspended in glucose medium. Conditions and details as described in Fig. 1. Curve 1, controls in 5.5 m*M* glucose with no additions (14 atria); curve 2, pyruvate (9 atria); curve 3, acetate (10 atria); and curve 4, butyrate (8 atria).

curve 5), a nonmetabolizable monosaccharide, had no significant effect on the magnitude of atrial developed tension. Similar results were obtained with 5.5 or 11.0 m*M* sucrose. However 11.0 m*M* 3-*O*-methylglucose, resulted in a significant negative inotropic response (Fig. 2, curve 5).

The positive inotropic effect produced by the addition of glucose does not appear to be related to a release of catecholamines. The stimulation of developed tension by glucose occurred in atria isolated from reserpinized animals (5 mg/kg/24 hr) as well as in atria exposed to tyramine to the point of tachyphylaxis. Also, a reduction of cholinergic activity is not the mechanism for the positive inotropic effect for the stimulation of developed tension by glucose occurred in atropinized atria (0.01 m*M* atropine). Inasmuch, as 5.5–11.0 m*M* sucrose did not produce a significant positive inotropic response it is unlikely that the effect of glucose on atrial developed tension resulted from an elevation of the osmolarity of the medium.

Figures 3–5 summarize the inotropic effects of various concentrations of pyruvate, acetate, and butyrate. All of these substrates produced an initial transient depression of the magnitude of developed tension; this effect was followed by a secondary increase of developed tension, the magnitude of this increase differed with each substrate as well as with each concentration. Pyruvate at 2.75 m*M* produced a small but significant positive inotropic effect (Fig. 3, curve 2); at 5.5 m*M* had no significant effect (Fig. 4, curve 2) whereas at 11.0 m*M* a definite progressive decrement occurred (Fig. 5, curve 2). With acetate or butyrate no stimulation above the control levels was observed (Figs. 3–5, curves 3 and 4). It is evident that the effects of pyruvate, acetate, and butyrate differ from those obtained with increasing concentrations of glucose or mannose. It is also clear that the initial transient depression produced by acetate (Figs. 3–5, curve 3) or butyrate (Fig. 3–5, curve 4) was significantly greater than that elicited by pyruvate; hence the anionic part of these molecules appears to have a more specific influence on this initial depression than the accompanying cation, Na^+ (2).

The possibility that the negative inotropic effect observed after the addition of these

substrates were due to an elevation of the osmolarity of the medium was tested with isosmolar concentrations of sucrose. It was found that sucrose at these concentrations had no significant effects on the magnitude of atrial developed tension. Furthermore, the participation of cholinergic mechanisms do not appear to be significantly involved in these phenomena, for the negative inotropic effects of these substrates were also present in atropinized preparations (0.01 m*M* atropine).

Discussion. The results of this investigation demonstrate that the magnitude and stability of developed tension of isolated rat atria suspended in substrate-free medium or in the presence of 3-*O*-methylglucose, was significantly less than when glucose, mannose, fructose, lactate, acetate, butyrate, or pyruvate was the substrate. The rapid negative inotropic response observed in substrate-free medium as well as in the presence of the nonmetabolizable monosaccharide 3-*O*-methylglucose, suggest that the endogenous energy stores of rat atria are not adequate for the support of the processes involved in the maintenance of the developed tension magnitude.

Furthermore, glucose 11.0 m*M* was able to support the developed tension of a prolonged period of time without significant decrement. On the other hand, with 5.5 m*M* glucose as well as with 5.5 11.0 m*M* mannose, pyruvate, lactate, acetate, or butyrate a small but definite decrement occurred. It was also observed that increasing developed tension magnitude followed the addition of increasing concentrations of glucose. The addition of mannose also stimulated the developed tension but significantly less sustained than glucose. The 3-*O*-methyglucose not only failed to support atrial contractile tension but also did not produce a positive inotropic effect when 5.5 m*M* were added to a medium containing glucose. Furthermore the addition of higher concentrations (11.0 m*M*) were clearly depressant. This is in contrast to the contractile stimulation observed after the addition of metabolizable monosaccharides (9–11), *i.e.*, glucose and mannose. Inasmuch as 3-*O*-methylglucose is transported across the membrane of muscle cells with a similar kinetics as glucose but is not metabolized (12–13), it would appear that transport per se is not the primary mechanism by which the metabolizable monosaccharides can either maintain atrial developed tension or produce a positive inotropic effect.

As indicated above the stability of developed tension varied with the concentration of glucose. At 5.5 m*M* a small but definite decrement was observed, whereas at 11.0 m*M* the decrement was significantly less. Furthermore, the addition of increasing concentrations of glucose resulted in a sustained positive inotropic effect. Opie *et al.* (14) demonstrated in the perfused rat heart that when the concentration of glucose was elevated from 5 to 10 m*M* the rate of metabolism of this substrate was almost doubled. Therefore, it is likely that the effects of increasing concentrations of glucose on the magnitude and stability of myocardial developed tension, as presently observed on isolated rat atria, could be associated with an increased metabolic rate. Inasmuch as mannose is also transported (15) and metabolized (10–11) by muscle cells, it is reasonable that the inotropic effects of mannose are also associated to a metabolic mechanism simular to that of glucose.

Opie *et al.* (14) demonstrated in the perfused rat heart that the uptake and metabolism of fructose was significantly less than that of glucose and a similar finding has been made by Danforth and co-workers on the human heart (16). From these studies they concluded that it is unlikely that fructose could serve directly as a fuel for myocardial contraction. Our observation that fructose was not as effective as glucose, mannose, lactate, acetate, butyrate, or pyruvate for the maintenance of atrial developed tension magnitude is in agreement with the above findings. However, the fact that the contractile tension of rat atria suspended in the presence of fructose was higher and better sustained than in substrate-free medium or in the presence of the nonmetabolizable monosaccharide 3-*O*-methylglucose, indicate that fructose, even though less effective than glu-

cose, mannose, lactate, acetate, butyrate, or pyruvate, can support a definite level of atrial contraction. On the other hand the fact that increasing concentrations of fructose failed to stimulate atrial contraction as glucose or mannose, could also be associated with its comparatively reduced metabolism by the myocardium.

When the concentration-action relation of glucose was compared to those of 3-*O*-methylglucose, pyruvate, acetate, and butyrate, it was found that increasing concentrations of glucose resulted in a positive inotropic effect; whereas increasing concentrations of 3-*O*-methylglucose, pyruvate, acetate, and butyrate depressed the contractile tension. It is well known that pyruvate, acetate, and butyrate are readily oxidized by the perfused rat and guinea pig heart (5, 17–20) and we have demonstrated that pyruvate can elevate ATP levels of isolated rat atria (1). On the other hand it has also been shown that acetate, butyrate, and other sources of acetyl CoA markedly reduce the oxidation of exogenous glucose by the isolated heart and the suggestion was made that this resulted from an inhibition of pyruvate oxidation with no influence upon glucose uptake or glycolysis (20). Indeed, the finding that fatty acids as well as acetate increase the heart levels of acetyl-CoA which in turn can inhibit heart pyruvate dehydrogenase (21) is in favor of such a mechanism as the reason for the reduction of glucose metabolism. However, it was also observed that fatty acids, acetate, and pyruvate at high concentrations inhibited not only the oxidation of glucose but reduced its uptake by the perfused rat heart (5, 7). Suggestions were made that these influences are mediated by changes in the tissue levels of citrate as well as by changes in the ATP/ADP ratio which regulate the activity of the enzyme phosphofructokinase in the glycolytic sequence (4–7, 22–24). Therefore, it is reasonable that the negative inotropic effect observed with increasing concentrations of pyruvate, acetate, or butyrate could be the consequence of an inhibition of early steps in the metabolism of glucose. Also, it would appear from the present data that the stimulation of contractile tension produced by increasing concentrations of glucose or by the addition of mannose, was not merely the consequence of an increased formation of oxidizable substrates for the tri carboxylate cycle. On the other hand, inasmuch as the nonmetabolizable monosaccharide 3-*O*-methylglucose compete with glucose for transport across muscle cell membranes (11, 25), it is possible that the negative inotropic effect observed with 11.0 mM 3-*O*-methylglucose is also associated to a reduction in the uptake and metabolism of glucose by the atria.

In previous studies we found that the developed tension of isolated rat atria is particularly sensitive to the removal of glucose, decreasing very rapidly in glucose-free medium. The readmission of glucose is followed by a complete recovery of the control levels of developed tension (2, 3). Similarly, 2-DG, an inhibitor of glycolysis, exerted a negative inotropic effect; this was accompanied by a fall in the ATP content of the atria (1). The addition of increasing concentrations of glucose counteracted to some extent the contractile depression by 2-DG and partially restored atrial ATP levels. Pyruvate, on the other hand, completely restored the ATP concentration of atria made hypodynamic by incubation in glucose-free medium or by exposure to 2-DG, however it was significantly less effective than glucose in restoring the magnitude of developed tension. Similar results were obtained with lactate (2). From these studies it was suggested that the magnitude of atrial developed tension is influenced by some process that occurs during the initial phases of glucose metabolism. The findings of the present study are also in accord with this interpretation and again suggest that glucose and the operation of the Embden-Meyerhof pathway chain exerts a unique role in the regulation of the magnitude and stability of the developed tension of isolated rat atria.

Summary. The initial developed tension magnitude as well as the stability of the developed tension magnitude of isolated rat atria suspended in different substrates were

studied. The initial developed tension was significantly greater in the presence of glucose, mannose, lactate acetate, butyrate, or pyruvate than when fructose or the nonmetabolizable monosaccharide 3-*O*-methylglucose was the substrate. Atrial developed tension in fructose was significantly greater than in 3-*O*-methylglucose or in substrate-free medium. The stability of the developed tension was comparable in 5.5 m*M* glucose, mannose, lactate, acetate, butyrate, or pyruvate showing a small decrement with time (5–10% in 60–90 min period). Increasing concentrations of mannose, fructose, lactate, acetate, butyrate, or pyruvate up to 11.0 m*M* did not prevent the decrement of developed tension whereas it was completely abolished in the presence of 11.0 m*M* glucose. The ability of several substrates to modify the isometric contractile tension of rat atria suspended in KRB medium containing 5.5 m*M* glucose was also investigated. The addition of increasing concentrations of the metabolizable monosaccharides glucose and mannose, stimulate atrial developed tension, whereas fructose, had no effect. The 3-*O*-methylglucose had either no effect, or at higher concentrations resulted in a negative inotropic response. Increasing concentrations of butyrate, acetate, or pyruvate depressed the developed tension. It would appear that transport of sugars across the cell membrane or the increased formation of oxidizable substrates for the Krebs cycle are not "per se" the only mechanism by which the metabolizable monosaccharides can maintain atrial developed tension or produce a positive inotropic effect. The possibility that the magnitude of atrial developed tension is influenced by some process that occur during the initial phases of the glycolytic sequence is discussed.

The skillful technical assistance of Barbara Hewaldt and Berta Flores is gratefully acknowledged.

1. Gimeno, A. L., Lacuara, J. L., Gimeno, M. F., Ceretti, E., and Webb, J. L. Mol. Pharmacol. 2, 67 (1966).

2. Gimeno, A. L., Gimeno, M. F., Savino, E. A., and Bedners, A. S., Proc. Soc. Exptl. Biol. Med. 123, 875 (1966).

3. Savino, E. A., Gimeno, M. F., and Gimeno, A. L., Experientia 23, 144 (1967).

4. Newsholme, E. E., Randle, P. J., and Manchester, K. L., Nature 193, 270 (1962).

5. Williamson, J. R., Biochem. J. 93, 97 (1964).

6. Garland, P. B., Randle, P. J., and Newsholme, E. A., Nature 200, 169 (1963).

7. Williamson, J. R., J. Biol. Chem. 240, 2308 (1965).

8. Hohorst, H. J., *in* "Methods of Enzymatic Analysis" (H. U. Bergmeyer, ed.), Academic Press, New York (1963).

9. Bing, R. J., Physiol. Rev. 45, 171 (1965).

10. Park, C. R., Johnson, L. H., Wright, J. H., and Batsel, H., Am. J. Physiol. 191, 13 (1957).

11. Stein, M. W., J. Biol. Chem. 186, 753 (1950).

12. Morgan, H. E. and Park, C. R., Federation Proc. 17, 278 (1958).

13. Nagahara, H. T. and Ozand, P., J. Biol. Chem. 238, 19 (1963).

14. Opie, L. H., Shipp, J. C., Evans, J. R., and Lebocuf, B., Am. J. Physiol. 203, 839 (1963).

15. Levine, R., Goldstein, M. S., Huddlestun, B., and Klein, S. P., Am. J. Physiol. 163, 70 (1950).

16. Danforth, W. H., Hogancamp, C. E., Ballard, F. B., and Eing, R. J., Am. J. Med. Sci. 238, 477 (1959).

17. Williamson, J. R., Biochem. J. 83, 377 (1962).

18. Evans, J. R., Opie, L. H., and Renold, A. E., Am. J. Physiol. 205, 971 (1963).

19. Shipp, H. C., Mutos, O. L., Knizley, H., and Cravasse, L. E., Am. J. Physiol. 207, 1231 (1964).

20. Davis, E. J. and Quastel, J. H., Can. J. Biochem. 42, 1605 (1964).

21. Garland, P. B. and Randle, P. J. Biochem. J. 91, 6c (1964).

22. Passeneau, J. V. and Lowry, O. H., Biochem. Biophys. Res. Commun. 13, 372 (1963).

23. Williamson, J. R., Jones, E. A., and Azzone, G. F., Biochem. Biophys. Res. Commun. 17, 696 (1964).

24. Parmeggiana, A. and Bowman, R. H., Biochem. Biophys. Res. Commun. 12, 268 (1963).

25. Randle, P. J., *in* "Membrane Transport and Metabolism," (A. Kleinzeller, and A. Kotyk, eds.), p. 431. Academic Press, New York (1961).

Received Sept. 17, 1968. P.S.E.B.M., 1969, Vol. 130.

Effects of Oral Administration of Short-Chain Fatty Acids on Certain Blood and Urine Constituents of Fasted, Phlorhizin-Treated Ewes

G. D. GOETSCH, D.V.M., Ph.D., and W. R. PRITCHARD, D.V.M., Ph.D., J.D.

Lafayette, Indiana

Am. J. Vet Research 19; 637-41 (1958)

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[1] 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-depressive effect of the latter four acids. Johnson[2] and Clark and Mahon[3] 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,[1] and its antiketogenic effect was observed by Barnett and Porter.[4]

Phlorhizin is a naturally occurring glucoside which produces glycosuria in laboratory animals and dogs by blocking tubular reabsorption of glucose.[5] Its effects persist for several hour and, if administration is continued, a depletion of carbohydrate stores occurs, followed by hypoglycemia, ketonemia, and a marked increase in nitrogen excretion.[5]

In previous experiments,[6] 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.

From the Department of Veterinary Science, Purdue University, Lafayette, Ind. Submitted as journal paper No. 1185, Purdue University, Agricultural Experiment Station, Lafayette, Ind.

MATERIALS AND METHODS

Adult, native ewes (120), 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 18-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 18-hour fast and the period of phlorhizin treatment, and at zero, three, six, 24, and 48 hours after administration of the fatty acid. The blood sugar levels were determined by the Somogyi-Nelson[16] method, and blood ketone levels by the method of Barnes and Wick.[7] Urine was collected with a Foley 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

TABLE 1.—Effects of Water Administration on Fasted, Phlorizin-Treated, Nonpregnant Ewes.[a]

Hours	Blood Glucose	Blood Ketone	Urinary Glucose	Urinary Ketone
-48	[illegible]	[illegible]	[illegible]	[illegible]
-18	[illegible]	[illegible]	[illegible]	[illegible]
0	[illegible]	[illegible]	[illegible]	[illegible]
3	[illegible]	[illegible]	[illegible]	[illegible]
6	[illegible]	[illegible]	[illegible]	[illegible]
24	[illegible]	[illegible]	[illegible]	[illegible]
48	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

TABLE 2—Effects of Sodium Acetate Administration on Certain Blood and Urine Constituents of Fasted, Phloridzin-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.)
12	36.9	.....	.....	.....
0*	26.6 ± 1.6	4.2 ± 0.6	15.5 ± 5.4	13.5 ± [illegible]
1	23.9 ± 1.2	.....	.....	.....
3	22.7 ± 1.5	.....	.....	.....
6	22.8 ± 2.8	9.0 ± 0.7 ‡	.....	.....
24	23.1 ± 3.5	9.3 ± 0.7 ‡	19.0 ± 4.9	15.7 ± [illegible]
48	22.8 ± 1.2	12.7 ± 2.0	13.2 ± 6.7	14.1 ± [illegible]

* Drinking and phloridzin (2 Gm. twice daily) treatments were begun; sodium acetate was given at a dosage of 1 gram per 100 lb. of body weight. ‡ Indicates a significant difference at the 2 [illegible] level. Indicates a significant difference at the [illegible] level, for differences in paired samples, except that fasted ketone values were compared to those observed in the water-treated controls.

0.25 c.d. The 24-hour samples were pooled, and aliquots and either urea or total nitrogen determinations were made. Urinary glucose levels were determined by the method of Benedict,[7] urinary urea nitrogen by the method of Folin-Youngburg,[4] and total urinary nitrogen by the Koch-McMeekin[6] 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 gram and 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, 15.9 Gm. Urea nitrogen excretion appeared to decrease

TABLE 3—Effects of Sodium Propionate Administration on Certain Blood and Urine Constituents of Fasted, Phloridzin-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*	25.9 ± 2.3	4.9 ± 0.8	36.5 ± 5.3	11.4 ± [illegible]
1	19.1 ± 7.91 ‡	.....	.....	.....
3	29.7 ± [illegible]	.....	.....	.....
6	31.8 ± 5.4 ‡	2.1	.....	.....
24	40.8 ± 2.1 ‡	2.9 ± 0.1 ‡	69.2 ± 6.9 ‡	11.8 ± [illegible]
48	28.9 ± [illegible]	3.0 ± 1.5 ‡	55.2 ± 3.0	11.8 ± [illegible]

* Drinking and phloridzin (2 Gm. twice daily) treatments were begun; sodium propionate was given at a dosage of 1 gram per 100 lb. of body weight. ‡ Indicates a significant difference at the 1 [illegible] level. Indicates a significant difference at the [illegible] level, for differences in paired samples.

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 ± 2.8	18.0 ± 1.1	20.5 ± 2.7
1	18.7 ± 1.8 §	15.0 ± 0.3 §	.....	.....	.....
3	16.5 ± 1.6 §	21.1 ± 2.1 §	.....	.....	.....
6	19.0 ± 1.7 §	22.9 ± 2.6 §	.....	.....	.....
24	27.7	17.8 ± 2.1 §	31.1 ± 2.6 §	9.5 ± 1.0 §	18.2 ± 2.3
48	26.0	12.5 ± 3.0	35.6 ± 3.1 §	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 per 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.[5]

The degree of hyperglycemia observed in these trials resembles that observed in goats by Schultz and Smith[11] 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 concentration 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

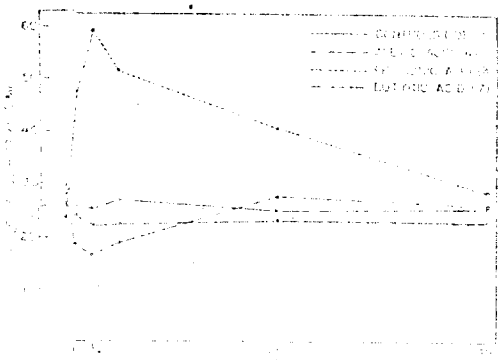


Chart. — Effects of oral sodium butyrate on certain blood constituents of fasted, phlorhizin-treated ewes. Fasted for 48 hours.

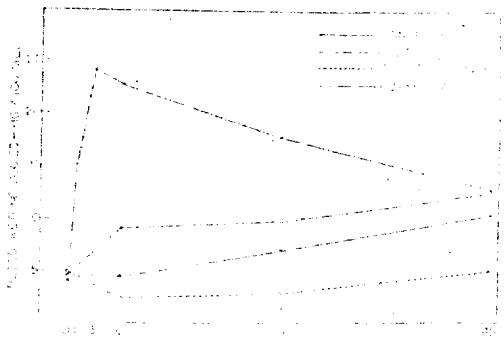
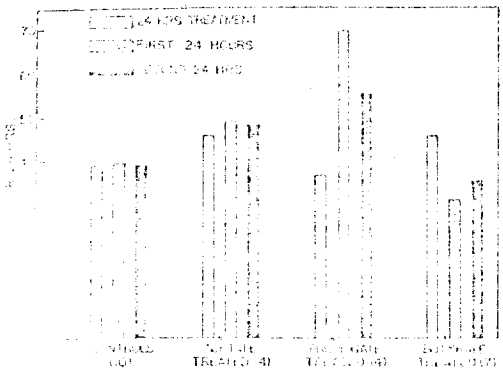


Chart. — Effects of oral sodium butyrate on certain blood constituents of fasted, phlorhizin-treated ewes. Fasted for 48 hours.



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[13] 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, seems to be correlated with its gluconeogenic 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 arteriovenous studies on 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.

References

[1] Barnes, R. H., and Wick, A. N.: A Method for the Determination of Blood Acetone Bodies. J. Biol. Chem., 177, (1949): 415.

[2] Benedict, S. R.: The Detection and Estimation of Glucose in Urine. J. Am. M. A., 57, (1911): 1193-1194.

[3] Clark, R., and Malan, J. R.: Alterations in the Blood Sugar and Ketone Levels by Dosing Acetate, Propionate and Butyrate into the Rumen of the Sheep. Onderstepoort J. Vet. Res., 27, (1956): 161-169.

[4] Folin, O., and Von Dam, W.: Notes on the Determination of Urea in Urine by Direct Nesslerization. J. Biol. Chem., 83, (1929): 111-112.

[5] Goetsch, G. D.: Studies on the Production and Treatment of Experimental Ketosis in Ruminants. Ph.D. Thesis, Purdue University, Lafayette, Ind., 1957.

[6] Marrett, L. G., and Porter, R. J.: Stereoisomers of Acetate and Propionate in the Ruminant. Science, Feb. 1956: 316-317.

[7] Johnson, R. B.: The Effects of Oral Administration of Acetic, Propionic and Butyric Acids Upon the Blood Glucose and Ketone Body Levels of Goats. Cornell Vet., 45, (1955): 275-290.

[8] Johnson, R. B.: Ruminant Ketosis. Vet. Scope, Upjohn Co., pub., (1956): 2-10.

[9] Kidd, P., Marshall, R. A., and Phillipson, A. T.: A Comparison of the Mixtures of Acetic, Propionic and Butyric Acids in the Rumen and in the Blood Leaving the Rumen. J. Exp. Biol., 28, (1951): 207-217.

[10] Kolb, E. G., and McMillan, R. L.: A New Direct Method for Blood Acetone Bodies. J. Am. Chem. S., 57, (1935): 2079-2080.

[11] Pennington, R. J.: The Metabolism of Short-Chain Fatty Acids in the Sheep. 1. Fate and Fermentation of Acetic Acid in the Rumen. Biochem. J., 57, (1954): 380-391.

[12] Reid, R. L.: Studies on the Carbohydrate Metabolism of Sheep. II. The Uptake by the Tissues of Glucose and Acetic Acid from the Peripheral Circulation. Austral. J. Agric. Res., 1, (1950): 338-354.

[13] Schultz, L. H., and Smith, A. [illegible] the Alimentary Tract of Goats [illegible] Ketone Bodies in Ruminants. J. Dairy Sci., 41, (1958): 817-822.

[14] [illegible] Society of Research [illegible] of Rumen Bacteria to Ketosis. J. [illegible] (1951): [illegible].

[15] [illegible] A Manual of Clinical [illegible] W. B. Saunders Co., Philadelphia, [illegible]

[16] Somogyi, M.: Notes on Sugar Determination. J. Biol. Chem., 195, (1952): 19-23.

J. Cellular Comp. Physiol 23: 247-66

THE RELATIVE RATE OF PENETRATION OF THE LOWER SATURATED MONOCARBOXYLIC ACIDS INTO MAMMALIAN ERYTHROCYTES

JAMES W. GREEN*

Physiological Laboratory, Princeton University and The Marine Biological Laboratory, Woods Hole, Massachusetts

FOUR FIGURES

Since the work of Overton (1895, 1899, 1902) the idea has been widely accepted that compounds soluble in lipid solvents penetrate cells by reason of their solubility in the lipids of the cell surface. A number of investigators (Loeb, Harvey, Crozier, Jacobs, Bodansky, Collander and Bärlund, Höber and Orskov, Dziemian) have tested this theory and, in general, the relative rate of penetration of the lipid soluble compound has been found to parallel its distribution in an oil-water mixture. These investigators used various methods to measure penetration of lipid soluble compounds, not all of which are equally reliable. Thus Loeb ('99, '13) using Arbacia eggs, studied the rate of fertilization membrane formation on exposure to the lower fatty acids. He found that as the fatty acid chain was lengthened, the membranes formed more rapidly, and considered his observations to support the Overton theory. However, in the absence of information showing that penetration must occur before membrane formation can take place, the Loeb results are best considered inconclusive support for the theory. Harvey ('44) and Crozier ('16) studied the penetration of acids into tissues of two invertebrate forms

*Present address: Department of Physiology and Biochemistry, Rutgers University, New Brunswick, N.J.

which contained natural indicators. A color change in the indicator was taken as evidence of penetration of the acid. Both investigators found valeric or isovaleric acid to penetrate more rapidly than lower acids.

The studies of Jacobs ('26), Bodansky ('28), Höber and Orskov ('32) and Dziemian ('39) were made on erythrocytes using hemolysis as a criterion of penetration. This method has many advantages when used critically but, as will be shown by this report, cannot be accepted as a measure of penetration of compounds which enter cells more rapidly than water, without reservation. Of this group of hemolysis studies only those of Jacobs and Bodansky were concerned chiefly with the penetration of fatty acids into erythrocytes. Jacobs, using the ammonium salts of the fatty acids from formic to valeric, was able to show an increasing rate of penetration for the series. This is in agreement with the Overton theory. Bodansky studied the hemolytic effects of the fatty acids themselves. However, he neglected to distinguish between hemolysis wrought by injury and that produced by penetration of the acid and water and it is difficult to evaluate his results. The work of Collander and Bärlund ('33) dealt with the penetration of lipid soluble compounds in the cells of the large alga, Chara. They determined the amount of a compound which had entered the cell by chemical analysis and found for the most part agreement between the rate of penetration and the lipid-solubility of the compound. They did not study fatty acid penetration.

The present study has attempted to measure the relative rate of penetration of the lower fatty acids into mamalian erythrocytes by a new method. This method is essentially chemical and depends upon the fact that oxyhemoglobin when placed in an environment containing an increased hydrogen ion concentration loses some of its oxygen. This loss of oxygen may be determined spectrophotometrically. Under experimental conditions the rate at which oxyhemoglobin is converted into reduced hemoglobin in the intact erythrocyte in a fatty acid environment may be used as a measure of the rate

TABLE 1

Acid	Concentration of acid in water in per cent	Refractive index of acid in water at 20° C.	Acidity of mixture in equivalents per liter	pH of mixture at equilibrium	Concentration of acid in mixture in per cent	HB_{50}[2]	Percentage HB_{50}[3]
Formic	0.006	0.003	4.31	4.6x/1	72.51	8.55	0.005 (0.1 N acid)
Acetic	0.003	0.004	4.18	0.282/1	70.78	12.93	0.93 (0.1 N acid)
Propionic	0.01	0.005	4.26	0.257/1	66.21	17.52	0.1515 (0.01 N acid)
Butyric	0.01	0.005	4.28	0.282/1	56.58	22.05	0.113 (0.01 N acid)
Valeric	0.009	0.0015	4.26	0.291/1	65.46	26.3	[illegible] (0.01 N acid)
Caproic	0.01	0.005	4.27	0.257/1	55.58	30.75	9.47 (0.01 N acid)
Heptylic	0.008	0.004	4.39	0.358/1	40.25	35.7	19.3 (0.01 N acid)
Caprylic	0.003	0.0015	4.88	1.09/1	28.82	39.7	31.9 (0.001 N acid)

[1] Measured again 1 hr.
[2] Mean hemolysis for 30 hrs. [3]
[3] Ratio for acid in olive oil to acid in water at equilibrium (Bodansky, 29).

proximate concentration obtaining in the red cell suspension upon dilution in the rapid mixing device. The acid concentration used was determined by experiment as that concentration which upon mixing with the red cell suspension would yield a pH of approximately 4.2. At this pH the erythrocytes were not immediately hemolyzed and the hydrogen ion concentration of the environment had been appreciably increased. Caprylic acid solutions were used at a slightly higher pH because of their lytic action and slight solubility.

It was found experimentally and could be expected from an examination of the oxygen dissociation curve that for a given hydrogen ion concentration a greater amount of oxyhemoglobin dissociated at oxygen tensions below 60 mm of Hg than at oxygen saturation. In practice beef red cell suspensions and acid solutions were equilibrated to calculated oxygen tensions of 10 mm Hg and human red cells suspensions to 20 mm Hg. The equilibration was accomplished by mounting two rotating tonometers on the laboratory table beside the spectrophotometer. Water displaced the gas mixture by means of a siphon and drove the gas through the tonometers from which it was carried through the reservoirs of the rapid mixing device and finally through a water trap. After equilibration, which was usually accomplished within 25 minutes, the red cell suspension and the acid solution, each of approximately 100 ml, were passed directly into the reservoirs without contact with air. The tonometers were not placed in a constant temperature bath but the room in which the equilibration was done maintained a fairly constant temperature. During any one experiment the temperature was never observed to vary more than 0.5° C. and throughout the period of this study the room temperature ranged from 20° to 23° C.

Following equilibration and transfer of the red cell suspension and acid solution to the reservoirs of the rapid mixing device, a "run" was made. This consisted of 5 or 6 different observations of flow and static transmission values at different reaction times. The mixing distance was maintained constant during a run and the reaction time was varied

by changing the speed of flow of the liquids through the observation tube. In a series of three runs at the same reaction time the difference between flow and static readings, measured as densities (in which the optical density is the reciprocal of the logarithm of the per cent light transmitted), were averaged. These average values were then corrected to a scale of 100 in order to make the different series comparable. Control studies were made using 1% NaCl solutions in place of the acid stream, in order to determine instrument variation effects of change of velocity of flow and turbulence. The effects of light scatter were also investigated and considered of negligible importance in this study. When a red cell suspension was made up in 1% NaCl solution and a series of readings on the suspension made, it was found that consecutive readings did not vary more than 0.5%. Concentrations of red cells were used which transmitted approximately 50% of the incident light. Under these conditions, approximately 5% difference in transmission of light occurred between the absence of penetration of an acid and maximum penetration.

It was of importance to this study to know when hemolysis began and something of its course in time. Also because hemolytic techniques had been used as a measure of the penetration of compounds into red cells it was of interest to compare hemolytic data for these acids with the penetration data obtained by the spectrophotometric technique. Therefore, studies with the densimeter apparatus of Parpart (25) were made for each acid and each type of blood studied using the same acid and blood concentrations as were used in the rapid mixing device.

RESULTS

A summary of typical results obtained is shown in figure 2 for beef blood and figure 3 for human blood. These graphs are semi-logarithmic plots in which the lines were fitted by the method of least squares. Each point on a line is an average of three measurements. The ordinate, corrected density difference, is plotted against time in seconds. The time

referred to is the reaction time previously described. Density differences were obtained by subtracting static density values from flow density values. An average of three such differences for a given reaction time was corrected on the basis of 100 in order to bring the results for different acids on one graph. The failure of the lines for the different acids to have a common origin in both figures 2 and 3, is owing to their having been calculated lines. While the exponential nature of these data may be expected from theoretical considerations,

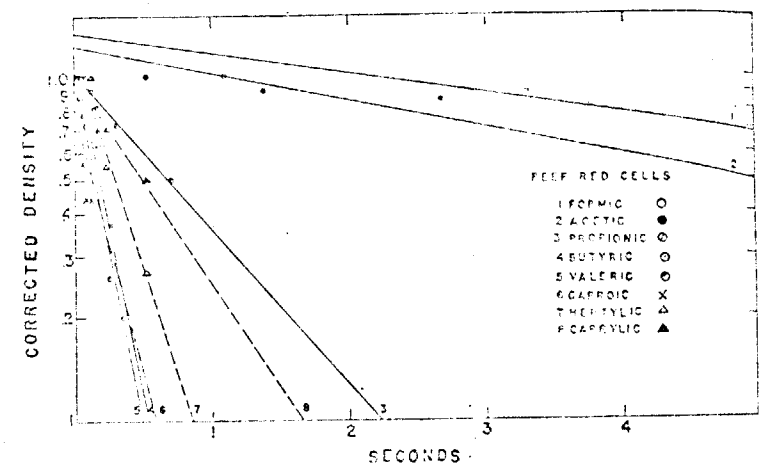


Fig. 2 Semi-logarithmic plot of representative data for beef erythrocytes. The ordinate, corrected density, refers to density differences between flow and static readings in the apparatus.

it was confirmed by plotting the corrected density difference against the reaction time. For convenience of presentation the semi-logarithmic plot only is given here.

Figure 2 shows a progressive increase in the rate of penetration of the acids into beef cells from formic to valeric, with butyric, valeric and caproic acids having much the same slope. The slopes of these three acids are not statistically different. A reverse condition was obtained for the rate of penetration of heptylic acid which was slower than caproic while caprylic

300

acid is slower than heptylic. Figure 3, for human blood, shows much the same trend as figure 2 for beef blood. The exception to be noted is that caprylic acid has a slightly slower rate of penetration than does heptylic acid. Since the lipid solubility of these acids increases with the length of the carbon chain, the results obtained with heptylic and caprylic acids were sufficiently in disagreement with the Overton theory to suggest a confirmatory study. This was undertaken with a Beckman spectrophotometer which has an altogether different optical and recording system from that of the Coleman 18S.

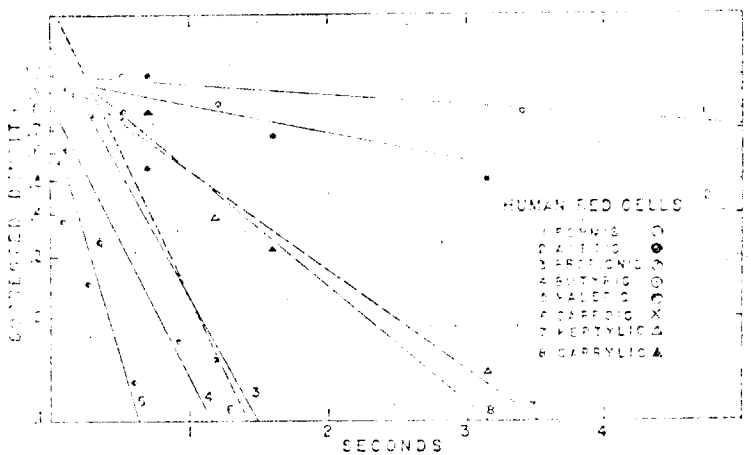


Fig. 3. Semi-logarithmic plot of representative data for human erythrocytes.

The same results were obtained with the Beckman instrument using beef blood only.

The results of the densimeter studies, which were performed for each acid and each type of blood, are plotted in figure 4. These graphs compare time to 50% hemolysis, using the densimeter technique, with time to 50% penetration, using the spectrophotometric technique. The data for the penetration plots were calculated from the relation, $\log C/C_\infty = -kt$, where C_∞ is the final concentration of acid at equilibrium inside the cells, C, the concentration at any given time, and

$-k$, the slope of the plot of $\log C/C_0$ against t, the time in seconds. When $C_\infty = 50$, $C_0 = 100$, and $t = 50$, the following relations exist: $t_{50} = 0.3010/-k$. An examination of figure 4 reveals that the beef and human red cells produce parallel curves for the hemolytic data and parallel curves for the

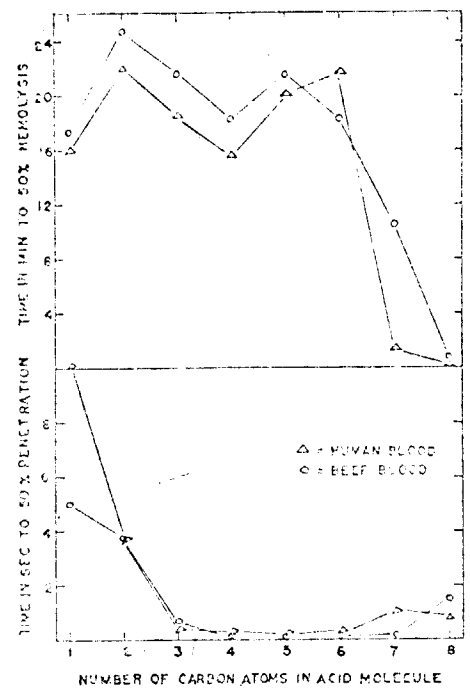


Fig. 4. Comparison between hemolytic and penetration times for each of the acids studied. The hemolysis scale is in minutes and the penetration scale in seconds.

penetration data. However, the two sets of curves are roughly the inverse of each other. In the hemolytic curves, formic acid produces 50% hemolysis about as rapidly as butyric, and one might expect that valeric would be faster than either but this is not the case. In the penetration data the most unexpected result is the decrease in the rate of penetration with

hexoylic and caprylic acids when these are known to be the most lipid soluble of the group studied. The times to 50% penetration are listed in table 2 for each acid and each type of blood studied.

TABLE 2

Time to 50% penetration

ACID	BEEF CELLS $-k^{1}$	BEEF PENETRATION TIME 50% PENETRATION	HUMAN CELLS $-k^{1}$	HUMAN PENETRATION TIME 50% PENETRATION
Formic	0.05569	5.04	0.02564	10.13
Acetic	0.07934	3.79	0.05073	5.73
Propionic	0.5534	0.66	0.7316	0.412
Butyric	2.0816	0.155	0.7949	0.379
Valeric	2.580	0.155	1.3872	0.175
Caproic	1.5312	0.197	0.8972	0.359
Hexoylic	1.2558	0.250	0.2595	1.04
Caprylic	0.6375	0.506	0.3924	0.878

[1] $-k$ = slope of plot from figure 2.

DISCUSSION

A number of investigators have studied the penetration of the lower fatty acids into cells and a few have determined the relative order of penetration of these acids. Harvey ('14a, '14b), taking advantage of a natural indicator in the pigment cells of the holothurian, Stichopus, observed the following order of penetration of the lower fatty acids: valeric = formic > butyric = propionic = acetic. Crozier ('16a, '16b) reported similar results using pigment cells in a mollusc, Chiton ulvoris. He obtained the series: isovaleric > formic > butyric > propionic > acetic. Crozier later ('18) determined the effect of these acids on the sensory activation of the earthworm, Allolobophora, but these results, like those obtained earlier by Loeb ('02, '13) on sea urchin fertilization membrane formation or those of Taylor ('28) on taste threshold of acids in human beings, are difficult to assess because of the absence of evidence for penetration.

Gryns (1896) observed that the ammonium salts of acetic, propionic and butyric acids penetrated horse erythrocytes. Later Jacobs ('25, '27) measured the hemolysis times of ammonium formate, acetate, propionate, butyrate and valerianate in beef erythrocytes. He found an increase in the rate of hemolysis with increasing length of the carbon chain, although some leveling off in the speed of penetration was observed in the butyrate and valerianate salts. He advanced evidence that the hemolysis was produced by the penetration of ammonia and fatty acid molecules following hydrolysis of the salt. Subsequently Jacobs and Parpart ('38) and Jacobs ('40) were able to show that at extreme pH values, either acid or alkaline, the hemolysis was slower than in the neutral range when larger numbers of both species of molecules were available for penetration. Höber ('36) made a study of the hemolysis rate of the sodium and ammonium salts of the lower fatty acids using the photocell method of Höber and Orskov ('23). He found, using red cells of the beef, sheep, rat, man and mouse, that exposure of these cells at pH 7.3 to solutions of the sodium salts of the C_2 to C_6 fatty acids resulted in no detectable alteration of the cells within many hours; however, the C_7 to C_9 fatty acids produced hemolysis within 20 minutes. With the ammonium salts of these acids he obtained the same results as did Jacobs. He further observed the hemolytic rate for the ammonium salts rose among the species studied in the following order: mouse, rat, man < beef < sheep. Dziemian ('39) who repeated this work in part, using the ammonium salts of the C_1 to C_4 fatty acids, was in agreement with it except for some of the species difference observations. He found the rat cells more permeable than the beef cells in every instance studied.

Stewart ('31) investigated the volume changes in sea urchin eggs when exposed to the same series of ammonium salts of fatty acids as Jacobs had studied in beef erythrocytes. She also found an increasing rate of volume change with increasing carbon content of the fatty acid and attributed the differential rate of penetration to the relative ease of solubility

of these acids in the lipids of the egg surface. Bonilleme in 1931 made a study of the penetration of the ammonium salts of the lower fatty acids into epidermal cells of Tradescantia virginiana using a deplasmolysis technique. He observed the same order of penetration as had Jacobs and Stewart. In these studies of Jacobs, of Höber, of Stewart and of Bonilleme, the penetration of ammonia molecules must be regarded as a contributing factor in the volume change or hemolysis observed. And while there is no reason to believe that in the presence of ammonia the order of penetration of these acids could be changed, the rate of hemolysis or volume change may be modified. Bodansky ('28, '31, '32, '33) investigated this point in dog and human erythrocytes using solutions of the lower fatty acids in physiological salt solutions. With complete hemolysis as a criterion of penetration rate, he observed differences in rate of penetration from C_2 to C_{10} fatty acids. He correlated these findings with the relative solubility of these acids in olive oil and associated their penetration into cells with lipid solubility. Solutions of the acids C_2 to C_6 were used at a pH below 4.0; the solutions of C_7 to C_{10} acids were intensely lytic as shown by Höber ('26). This work of Bodansky's appears to involve what Jacobs ('34) called secondary penetration of acids or penetration with injury. His work does raise the question as to to what extent hemolysis may be used as a criterion of penetration of a lytic compound. The work of Parpart and Shull ('35) indicated that in studies with nonlytic compounds such as glycerol, volume changes are closely paralleled by entrance of the glycerol. In the present study a comparison was made between rates of penetration as determined by hemolysis, using the densimeter technique of Parpart ('35) and penetration as determined more photometrically by the modified Hartridge-Roughton technique. The results are shown in figure 4. The irregularity in the hemolysis curve for the C_4 acid may be owing to evaporation of fluid in the densimeter. However, the hemolysis curves bear no resemblance to the penetration curves, although aside from instrumental differences both curves were

obtained under the same conditions. This graph indicates that rates of hemolysis of red cells by lytic compounds do not parallel rates of penetration of these compounds as determined by the modified Hartridge-Roughton technique.

The lower graphs of figure 4 show clearly the results of this investigation on beef and human cells. An increase in rate of penetration occurs between C_4 and C_5 acids with C_4, C_5 and C_6 acids exhibiting a nearly constant rate of penetration. The C_2 and C_3 acids penetrate more slowly than the C_4 to C_6 acids. The fact that these studies were made at the pH range 4.2 to 4.8 rather than at neutrality may have influenced the results. However, it was technically impossible to conduct the experiments at pH 7.0 and in any case, the measurements were made in such short periods of time that the lytic action of the higher acids was minimized. In addition to presenting information on the relative rate of penetration of these acids the time to 50% penetration has been calculated and has been found to be quite rapid, varying from 0.14 seconds to over 5.0 seconds as shown by table 2. The technique used in the present study is thought to yield less equivocal data on the penetration of these acids than any of the other methods used in this type of investigation. The fact that the present study has been limited to the blood of two species of mammals and has been made at approximately a single pH value limits the general applicability of the results. However, the results obtained, when their limitations are considered lend only partial support to the published evidence for a continuous series of fatty acids whose rate of penetration into red cells increases with increasing carbon content of the fatty acid.

Some of the physical characteristics of the acids studied in this investigation are listed in table 1. An attempt has been made to correlate the penetration results of this study with some of these characteristics, particularly with concentration, molecular volume or distribution ratio. The C_2 to C_5 acids do appear to penetrate in the order of their relative solubility in olive oil but not in proportion to their solubility. The rates of penetration of the C_6 to C_8 acids do not follow

their relative solubility in olive oil. These data on penetra-
tion do not consistently favor the idea that these acids penetrate by reason of their solubility in lipid regions of the erythrocyte and in this respect support the findings of Dale-
man (1930). They do suggest that molecular size, in the case of the C_6, C_7 and C_8 acids, may be a limiting factor in the rate of penetration. And it is suggested that the plateau observed in butyric, valeric and caproic acids may represent a balance of two opposite forces, increasing molecular volume, on the one hand, tending to retard penetration and increased solubility in lipid regions, on the other hand, tending to increase the penetration rate.

In considering the significance of the results of this investigation, the fact should be noted that the times for penetration of these acids were measured and were found to be, by comparison with hemolytic times, of a much smaller magnitude. Hence work with the method described in this paper should lead to information leading to the determination of permeability constants for these acids.

SUMMARY

1. A modification of the Hartridge-Roughton rapid mixing apparatus was developed and used in conjunction with a spectrophotometer to measure the relative rates of penetration of lower fatty acids into beef and human erythrocytes. The measurements of penetration depended upon the experimental observation that oxyhemoglobin dissociates to a new equilibrium with an increase in the hydrogen ion concentration within the cell.

2. The relative rates of penetration of the fatty acids investigated were found to be (a) for beef cells: caprylic < heptylic < caproic = valeric = butyric > propionic > acetic > formic; (b) for human cells: caprylic = heptylic = caproic < valeric > butyric > propionic > acetic > formic.

3. The rates of hemolysis by these acids were determined and found to be (a) for beef cells: caprylic > heptylic > caproic > valeric < butyric > propionic > acetic < formic; (b) for human cells: caprylic > heptylic > caproic < valeric < butyric > propionic > acetic < formic.

4. The times to 50% penetration of these acids were measured and found to be of much smaller magnitude than those determined by hemolytic methods.

5. The results of penetration and hemolysis are discussed in the light of previous hemolytic work done with these acids and the more general studies made of penetration into eggs and tissue cells.

6. An attempt was made to correlate the penetration results with lipid solubilty, surface tension or molecular volume data. The retardation of penetration observed in the C_6, C_7 and C_8 acids suggest that in this homologous series in which lipid solubility increases with increasing carbon content of the acids, molecular volume remains a limiting factor in the penetration rate.

The author deeply appreciates the advice, suggestions and help given by Dr. A. K. Parpart during the course of these experiments.

LITERATURE CITED

Barcroft and Murray 1922 Observations upon the effect of high altitude upon the physiological processes of the human body. Appendix II. Note on the relative effects of carbonic and hydrochloric acids on the affinity of haemoglobin for carbon monoxide. Philosophical Trans. Roy. Soc. B., *211:* 465.

Bodansky, M. 1928 Lipoid solubility, permeability and hemolytic action of the saturated fatty acids. J. Biol. Chem., *79:* 241–255.

——— 1931 Relation of hemolysis to the primary penetration of fatty acids through the red cell membrane. Proc. Soc. Exp. Biol. and Med., *28:* 672.

——— 1932 The hemolytic action of fatty acids. J. Cell. and Comp. Physiol., *1:* 429.

——— 1933 The permeability and hemolytic action of the fatty acids and some of their halogen derivatives. J. Exp. Biol., *10:* 59.

Bouillenne, R. 1930 Pénétration des sels ammoniacaux dans les cellules végétales. Compt. rend. soc. biol. T., *103:* 50–52.

GRIMMER, R., UND H. EMDEN 1932 Porus-Widerstandsmessungen an Zellen und Geweben, II Die Permeabilität für Nichtelektrolyte. Arch. f. Exp. Path., 167: 105-114.

GROSS, W. J. 1918a Cell penetration by acids. J. Biol. Chem., 35: 255.

——— 1918b Cell penetration by acids. II. Further observations on the Chromophore of Chromoderis edeni. J. Biol. Chem., 36: 247.

——— 1928 Sensory activation by acids. Am. J. Physiol., 85: 525-541.

HALDANE, D. L. AND J. H. AUSTIN 1935 Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide hemoglobin and methemoglobin. J. Biol. Chem., 114: 51-65.

HEMMING, A. J. 1930 The lipid content and permeability of the erythrocyte. J. Cell. and Comp. Physiol., 14: 193.

HERZ, G. 1931 Über den Einfluss gelöster Stoffe auf die roten Blutkörperchen in Verbindung mit den Erscheinungen der Osmose und Diffusion. Pflügers Arch., 227: 86-119.

HOLT, P. 1917 Beiträge zur Lichtabsorption des Oxyhämoglobins. Biochem. Z., Berlin, 81: 238-281.

HOLT, H., AND E. J. W. ROBERTSON 1923 A method of measuring the velocity of very rapid chemical reactions. Proc. Roy. Soc. A., 103: 176-184.

——— 1923 The kinetics of hemoglobin. II. The velocity with which oxygen combines with its combination with hemoglobin. Proc. Roy. Soc. A., 103: 185-190.

——— 1927 The kinetics of hemoglobin. III. The velocity with which oxygen combines with reduced hemoglobin. Proc. Roy. Soc. A., 117: 678-683.

HOWLAND, R. N. 1914a Cell permeability to acids. Science, 39: 949.

——— 1914b The permeability of cells for acids. Internat. Z. f. physik. Chem. Biol., 1: 463.

HUGGINS, R. 1936 The permeability of red blood corpuscles to organic acids. J. Cell. and Comp. Physiol., 7: 367.

HUGGINS, R., AND GIBSON 1938 Untersuchungen über die permeabilität gleichwertiger Anelektrolyten bei den roten Blutkörperchen verschiedener Tierarten. Pflüger's Arch., 241: 509.

HÜFNER, G. 1879 Ueber die Bestimmung des Hämoglobin und Sauerstoffgehaltes im Blute. Zeitsch. f. physiol. Chem., 3: 1.

JACOBS, M. H. 1924 Permeability of the cell to diffusing substances. General Cytology, University of Chicago.

——— 1926-27 The exchange of material between the erythrocyte and its surroundings. Harvey Lectures series, 22: 146.

——— 1940 Some aspects of cell permeability to weak electrolytes. Cold Spring Harbor Symp., 8: 30-38.

JACOBS, M. H., AND A. K. PARPART 1931 Osmotic properties of the erythrocyte. II. The influence of pH, temperature and oxygen tension on hemolysis by hypotonic solutions. Biol. Bull., 60: 95-119.

——— 1938 X. On the permeability of the erythrocyte to ammonia and the ammonium ion. J. Cell. and Comp. Physiol., 11: 175.

LOEB, J. 1909 Die Chemische Entwicklungsregung des tierischen Eies. Berlin.

——— 1913 Artificial parthenogenesis and fertilization. University of Chicago.

OVERTON, E. 1896 Über die osmotischen Eigenschaften der lebenden Pflanzen und Tierzelle. Vierteljahrsch. Naturforsch. Ges. Zurich, 40: 159-201.

——— 1899 Ueber die allgemeinen osmotischen Eigenschaften der Zelle, ihre Vermuthlichen Ursachen und ihre Bedeutung für die Physiologie. Vierteljahrsch. Naturforsch. Ges. Zurich, 44: 88-135.

——— 1902 Beiträge zur allgemeinen Muskel und Nerven Physiologie. Pflügers Arch. f. d. ges. Physiol., 92: 115-280.

PARPART, A. K. 1935 The permeability of the mammalian erythrocyte to deuterium oxide (heavy water). J. Cell. and Comp. Physiol., 7: 153-162.

PARPART, A. K., AND J. G. STOLL 1935 Permeability of the erythrocyte for glycerol. J. Cell. and Comp. Physiol., 6: 129-135.

RUBINSTEIN, D. L., AND H. M. RAVIKOVICH 1946 Absorption spectrum of hemoglobin in red cells. Nature, 158: 952.

STEWART, D. 1931 The permeability of the Arbacia egg to nonelectrolytes. Biol. Bull., 60: 152-170.

TAYLOR, N. W. 1928 Acid penetration into living tissues. J. Gen. Physiol., 11: 207-219.

Amer. J. Cardiol, 22(3): 360-9, 1968

Metabolism of Infarcted Heart Muscle During Tissue Repair*

SIGMUNDUR GUDBJARNASON, PH.D., WOLFGANG BRAASCH, M.D., CHARLES COWAN, M.D. *and* RICHARD J. BING, M.D., F.A.C.C.

Detroit, Michigan

THE MECHANISM of tissue repair in infarcted heart muscle consists of two parallel phases, the catabolic phase of degradation of dead and severely damaged heart muscle cells, and the anabolic phase of fibroblastic proliferation and synthesis of scar tissue replacing the necrotic muscle.

After an acute coronary occlusion there is a rapid response of the infarcted tissue to the injury. The reparative processes, as manifested by the incorporation of glycine-2-C^{14} into cellular subfractions from normal and infarcted heart muscle, have been associated with an early increase in nucleolar ribosomal RNA and protein synthesis.[1] These synthetic processes require energy and substrates that cannot be readily transported into the ischemic area. The primary question concerning tissue repair is that of energy production in the ischemic tissue, since the energy for synthetic, reparative processes must be made available in the infarcted and hypoxic muscle.

The purpose of this study was to examine alterations in metabolism of infarcted cardiac muscle, particularly with respect to pathways of energy production and the mechanism of the fatty degeneration or lipid accumulation in the ischemic tissue.

MATERIALS AND METHODS

ANALYSIS OF ENZYME PROFILE

Myocardial infarction was produced in 45 mongrel dogs (10 to 15 kg.) under pentobarbital anesthesia (25 mg./kg.) by ligation of several branches of the anterior and posterior descending coronary arteries, as described elsewhere.[1] The animals were killed by removing the heart from the anesthetized animal from 5 hours to 10 weeks after infarction. Three samples were obtained from the same left ventricle: (1) center of infarcted tissue, defined arbitrarily as one third of the infarcted area, constituting the center of the ischemic zone; (2) periphery of infarcted tissue, defined as two thirds of the infarcted area, representing the ischemic tissue between the normal muscle and center portion of the infarct; and (3) "normal" left ventricular muscle. An additional control group, consisting of 12 normal dogs without myocardial infarction, was studied. This group was used to examine possible changes in uninjured tissue of the infarcted left ventricle. The tissue (1 gm. wet weight) was homogenized in a glass Potter-Elvehjem tissue grinder and extracted three times with 0.1 M phosphate buffer, pH 7.2, containing 1 mM adenosine diphosphate (ADP), 10 mM glutathione and 20 mM $EDTA_2$ at 0 to 4°C.[2] The extraction was first carried out with eight volumes of buffer and centrifuged at 100,000 × g. The procedure was repeated twice with four volumes of buffer each time. The combined supernatants were used for the assay of enzyme activities.

Enzyme Activities Determined: The response of heart muscle to an alteration in the biochemical environment accompanying coronary occlusion was examined by determining the activities of enzymes in the uninjured, normal part of the left ventricle and in the periphery and center of the infarct. The following enzymes of the Embden-Meyerhof pathway and oxidative metabolism were used to express alterations in enzyme activities in the infarcted tissue: fructose-1, 6-diphosphate aldolase (Ald), glyceraldehydephosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), nicotinamide adenine dinucleotide (NAD-) and nicotinamide adenine dinucleotide phosphate (NADP-) specific isocitrate dehydrogenase (IDH) and glutamate oxaloacetate transaminase (GOT). Of these enzymes only NAD-IDH is a specific mitochondrial enzyme, whereas MDH, NADP-IDH and GOT have isoenzymes in both the cytoplasmic and the mitochondrial compartments. The activity of α-glycerophosphate dehydrogenase (α-GPDH) was also determined; this enzyme serves as a link between carbohydrate and

* From the Department of Medicine, Wayne State University School of Medicine, Detroit, Mich.

Address for reprints: Sigmundur Gudbjarnason, PH.D., Wayne State University School of Medicine, 1400 Chrysler Freeway, Detroit, Mich. 48207.

lipid metabolism and is furthermore involved in the transfer of hydrogen from the extramitochondrial to the intramitochondrial compartments.[3] The hexosemonophosphate shunt was represented by glucose-6-phosphate dehydrogenase (G-6-PHD) and 6-phosphogluconate dehydrogenase (6-PGDH).

The enzyme activities were assayed spectrophotometrically in a Beckman DU spectrophotometer, equipped with a thermospacer, a Gilford Model 220 absorbance indicator and a Model 210 automatic cuvette positioner. The determinations of enzyme activities were performed according to modified methods of Schimasek,[4] Delbrück et al.[5] and Bücher et al.[6] The assays were re-examined with respect to linear kinetics and optimal substrate conditions for dog heart muscle extracts, and, when required, modifications were made ensuring optimal assay conditions.

The change in absorbance was first recorded for 5 to 10 minutes in absence of substrate, in order to obtain a "tissue blank," and then the reaction was started by addition of substrate. The difference in reaction velocity before and after addition of substrate was the reaction rate in $\Delta A340$/min. used for computation of enzyme activity, which was expressed in micromoles of substrate converted to product/min./mg. protein of the heart muscle extract. The millimolar extinction coefficient employed was 6.22 for NADH or NADPH at 340 mμ using cells of 1 cm. light path.[7] A linear correlation was established between the reaction rate in $\Delta A340$/min. and the amount of extract used in the determination of enzyme activities. The total volume of reaction medium was adjusted to 3 ml. for all routine measurements.

The amount of extract per test varied, depending upon activity of the enzyme. The activities of GAPDH, LDH, MDH and GOT were determined in an extract diluted 1:100 with distilled water (without loss in specific activity), using 0.05 (LDH, MDH) or 0.10 (GAPDH, GOT) ml. per test. The activities of the other enzymes were determined in undiluted extract using 0.01 ml. (Ald., NADP-IDH), 0.05 ml. (α-GDH, NAD-IDH), or 0.50 ml. (G-6-PDH, 6-PGDH). The dilution of the extract in the test medium was large enough to eliminate possible interference by the EDTA of the extraction-medium.

PROTEIN

The protein content of the tissue extract was determined by the biuret reaction with the use of bovine serum albumin as a standard.[8]

ISOTOPE INCORPORATION IN VIVO

The incorporation of acetate-1-C^{14} (SA = 8.47 mc./mM) into lipids of normal and infarcted heart muscle was studied in vivo by injecting labeled acetate intravenously (30 μc./kg.) into the animals 46 hours or 10 days after infarction. Two hours after injection of the tracer the thorax was opened under pentobarbital anesthesia and biopsy samples were obtained rapidly from the normal and infarcted myocardium, quickly frozen in pre-cooled Freon and subsequently transferred to liquid nitrogen.

The tissue samples used for analysis of lipids were homogenized and extracted with chloroform-methanol (2:1 v/v)[9] washed and evaporated to dryness. The lipids were redissolved in chloroform-methanol (2:1) and applied in spots (10 to 20 μl.) on silicic acid thin-layer plates.[10] A double development procedure, employing first a polar (diethylether-benzene-ethanol-acetic acid 40:50:2:0.2) and then a nonpolar system (diethylether-hexane 6:94) was used for the complete separation of the main lipid classes, i.e., phospholipids (PL), monoglycerides (MG), fatty acids (FA), cholesterol (C), diglycerides (DG), triglycerides (TG) and cholesterol-esters (CE). After a brief development of the pattern in iodine vapor, the silicic acid in the regions corresponding to individual lipid fraction was scraped off the plates into separate counting vials and counted after addition of 10 ml. of toluene scintillator in a Tri-Carb liquid scintillation spectrometer.

ISOTOPE INCORPORATION IN VITRO

The in vitro oxidation of glucose-C^{14} to $C^{14}{}_{O_2}$ and incorporation of C^{14}-glucose into lipids of heart muscle homogenate was studied in an incubation medium consisting of 2.5 ml. of homogenate from normal or infarcted heart muscle obtained 48 hours after infarction, representing 500 mg. of tissue in 0.25 M sucrose, 0.1 M phosphate-buffer (pH = 7.2); 1.0 ml. of buffer containing 0.1 M. phosphate (pH = 7.4); 6.6 mM citrate, 3.3 mM $MgCl_2$ and 25 mM $NaHCO_3$; ATP in variable quantities, making final concentrations ranging from 1 to 15 mM; 0.1 ml. = 10 μc. of acetate-1-C^{14}, SA = 4.87 mc./mM or 10 μc. of glucose-1-C^{14} or glucose-6-C^{14} (SA adjusted to 4.14 mc./mM respectively); 0.25 M sucrose added to make a final volume of 4 ml.

The tracer was added at time zero and the incubation was carried out at 37° C. for 30 minutes. The reaction was terminated by addition of 2.5 ml. ice-cold 1.6 N. perchloric acid (PCA). The $C^{14}{}_{O_2}$ was absorbed on filter paper (1 × 1 cm.) that had been soaked in Hyamine. The reaction vessel was placed on ice for one hour and shaken every 10 minutes to ensure complete absorption of the $C^{14}{}_{O_2}$. The filter paper containing the $C^{14}{}_{O_2}$ was transferred to 10 ml. of toluene scintillator. The radioactivity was measured 24 hours later (in a Tri-Carb liquid scintillation spectrometer) with an efficiency of 65 per cent.

The precipitate was extracted three times at 0 to 4° C. with 0.3 N. PCA to remove the labeled precursor, and the lipids were extracted with chloroform-methanol (2:1) and processed as described.

DETERMINATION OF ATP AND LACTATE

Biopsy samples from infarcted, uninfarcted and normal left ventricular muscle were rapidly removed from the beating heart shortly before the animal was

to be killed, and immediately frozen in pre-cooled Freon® (dichlorodifluoro-methane) as described.

The samples (50 to 150 mg.) were transferred to liquid nitrogen and subsequently homogenized and extracted with 4% perchloric acid. Lactate was determined spectrophotometrically according to Hohorst,[11] and adenosine triphosphate (ATP) was determined according to Adam.[12]

Results

ENZYME PROFILE

The enzyme profile of normal left ventricular muscle is illustrated in Figure 1. After myocardial infarction there was a rapid decline in activities of oxidative and glycolytic enzymes in infarcted tissue. Oxidative enzymes reached the lowest level of activity in the center of infarcted tissue 10 days after infarction, with 8 to 13 per cent of normal activity (Fig. 2 and 3). Ten weeks after infarction there was some recovery of enzyme activities, with an increase up to 20 to 36 per cent of the activities observed in uninfarcted control tissue. The activity of α-GPDH (Fig. 3) reached the lowest level of all the enzymes examined (7.7 per cent of control tissue), 10 days after infarction; it also showed the greatest increase in activity 10 weeks after infarction—to 57.4 per cent of control tissue.

The glycolytic enzymes also decreased significantly in activity (Fig. 4). Aldolase and LDH decreased to 14 and 21 per cent of the activity observed in uninfarcted areas of the same ventricle. However, the activity of GAPDH was only reduced to 37.4 per cent of control 48 hours after infarction and increased to 48.4 per cent of uninfarcted control tissue 10 days after infarction.

In contrast to the diminished activity of glycolytic and mitochondrial enzymes, there was a significant rise in the activity of the HMP-shunt. The activities of G-6-PDH and 6-PGDH increased rapidly after infarction and reached a 34- and a 31-fold level, respectively, 10 days after infarction (Fig. 5). Ten weeks after infarction the activities had declined and approached normal levels, and nine months after the coronary occlusion the activity of G-6-PDH was still about three times higher in the scar tissue of the healed infarct than in the normal cardiac muscle. In the uninfarcted area of the injured heart muscle a significant increase was observed in the activities of G-6-PDH (Fig. 6). The activity of G-6-PDH was increased by 260 per cent 10 days after infarction, but diminished subsequently and reached normal levels 10 weeks after coronary occlusion.

OXIDATION OF LABELED GLUCOSE TO $^{14}CO_2$

The relative oxidation of glucose-1-C^{14} by normal and infarcted heart muscle homogenates from infarcted tissue is illustrated in Figure 7. The enhanced formation of $C^{14}O_2$ from glucose-1-C^{14} in infarcted tissue reflects a greater increase in activity of the HMP-shunt since both tissue samples were obtained from the same left ventricle and assayed in an identical manner. The limitations in using $C^{14}O_2$ production from C_1 and C_6 labeled glucose, respectively, as an indicator for HMP-shunt activity have been well

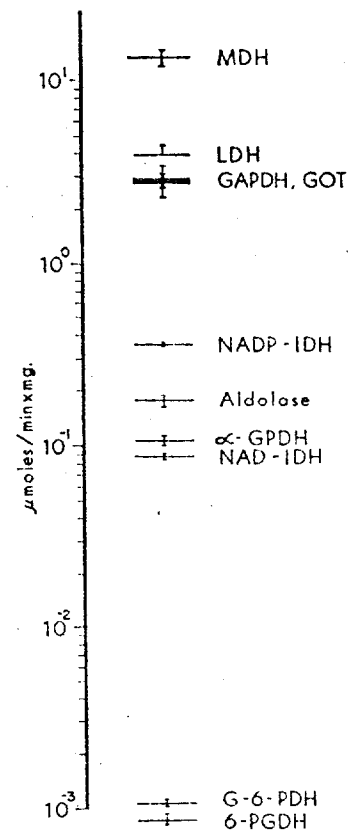


Figure 1. *The enzyme profile of dog heart muscle (left ventricle).* The specific activity of the enzyme was expressed in μmoles of substrate converted to product per minute per mg. of extracted protein. Malate dehydrogenase (MDH), 13.20 ± 1.31; lactate dehydrogenase (LDH), 3.84 ± 0.34; glutamate oxaloacetate transaminase (GOT), 2.86 ± 0.22; glyceraldehyde phosphate dehydrogenase (GAPDH, 2.85 ± 0.56; nicotinamide-adenine dinucleotide phosphate isocitrate dehydrogenase (NADP-IDH), 0.35 ± 0.0052; Aldolase (Ald), 0.176 ± 0.012; α-glycerophosphate dehydrogenase (α-GPDH), 0.1050 ± 0.005; nicotinamide-adenine dinucleotide-isocitrate dehydrogenase (NAD-IDH) (0.0866 ± 0.00046); glucose-6-phosphate dehydrogenase (G-6-PDH), 0.00103 ± 0.00004; 6-phosphogluconate dehydrogenase (6-PGDH), 0.00083 ± 0.00008).

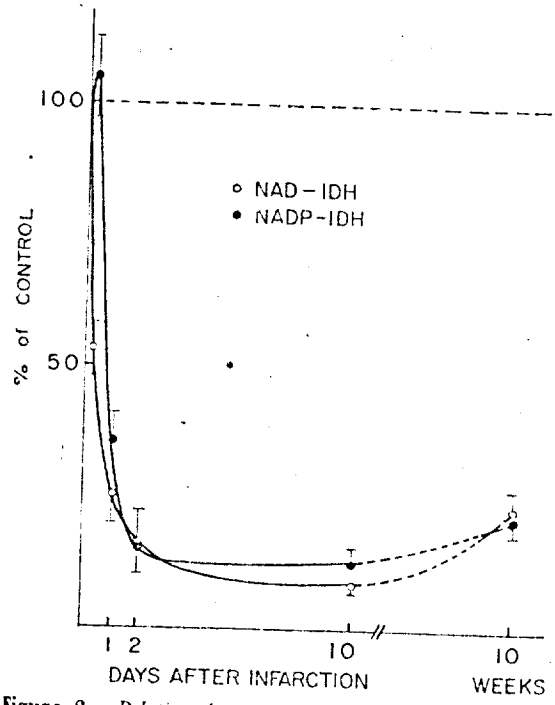


Figure 2. *Relative changes in the activities of NAD and NADP specific isocitrate dehydrogenases* (IDH) in infarcted heart muscle compared to uninfarcted tissue of the left ventricle.

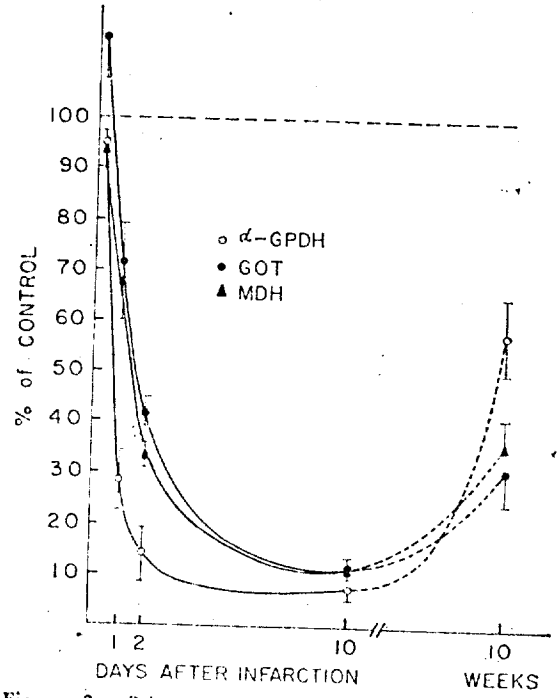


Figure 3. *Relative changes in the activities of α-GPDH, GOT and MDH* in infarcted heart muscle compared to uninfarcted tissue.

established.[13] However the order of change in oxidation of glucose-1-C^{14} to $C^{14}{}_{O_2}$ in infarcted tissue compared to uninfarcted muscle lends additional support to the observed increase in activities of HMP-shunt enzymes, although the uninfarcted tissue also demonstrates an increase in G-6-PDH activity compared to normal heart muscle without injury (Fig. 6).

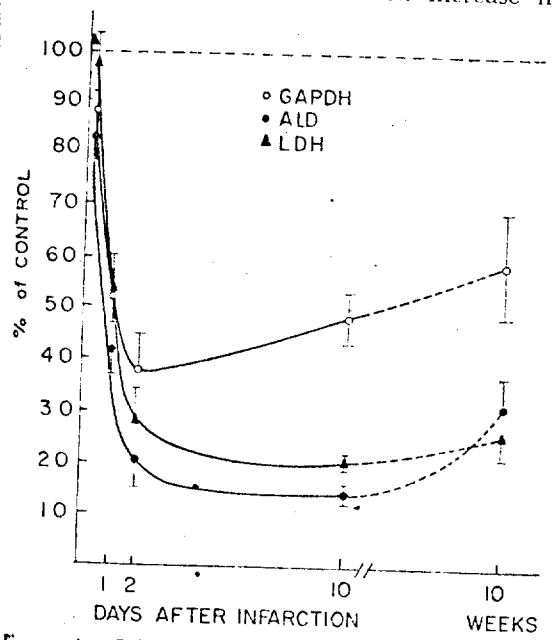


Figure 4. *Relative changes in the activities of GAPDH, Ald. and LDH* in infarcted heart muscle compared to uninfarcted heart muscle.

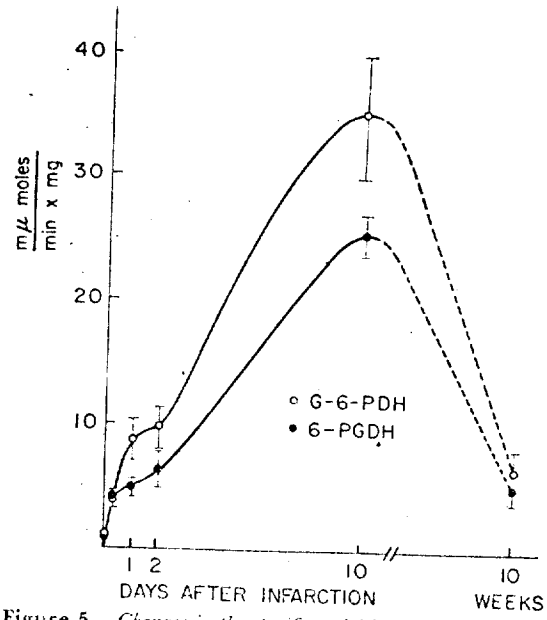


Figure 5. *Changes in the specific activities of G-6-PDH and 6-PGDH* in infarcted heart muscle as a function of time after coronary occlusion.

Our assay system did not contain exogenous NAD or NADP, but the effect of these coenzymes upon the C_1/C_6 ratio has been demonstrated in a study by Crevasse et al.[14,15] The increase in C_1/C_6 ratio from 3.5 in normal heart muscle homogenate to 9.8 in homogenate from infarcted tissue is of significance because both tissue samples were obtained from the left ventricle of the same heart and reflect alterations due to the coronary occlusion. Since the same reagents and tracers were used in both groups of experiments, a possible contamination of the labeled glucose with $C^{14}{}_{O_2}$ would not alter the results significantly.[16]

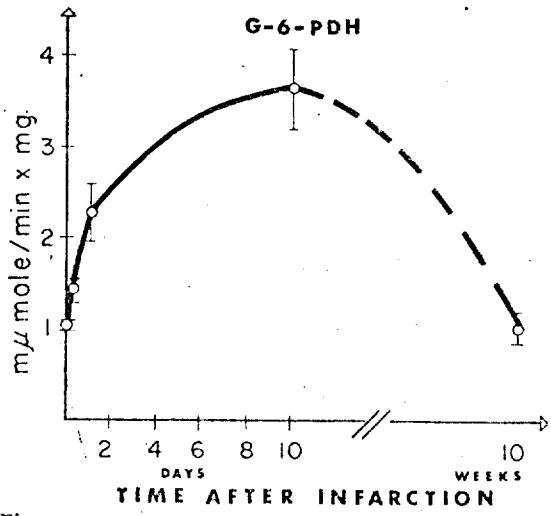


Figure 6. *Increase in G-6-PDH activity in uninfarcted areas of infarcted left ventricle* as a function of time after infarction.

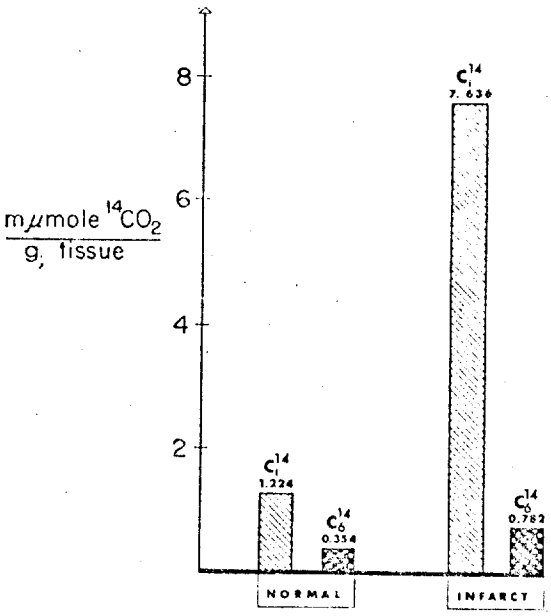


Figure 7. *Oxidation of glucose-1-C^{14} and glucose-6-C^{14} to $C^{14}{}_{O_2}$ by normal and infarcted heart muscle.* The incubation medium consisted of 2.5 ml homogenate representing 500 mg. of tissue in 0.25 M sucrose; 0.1 M phosphate buffer (pH 7.4); 6.6 mM citrate; 3.3 mM $MgCl_2$ and 25 mM $NaHCO_3$/0.1 ml. C^{14}-glucose = 10 μC.

LIPID SYNTHESIS

The incorporation of acetate-1-C^{14} into lipids of normal and infarcted heart muscle is illustrated in Table I. The incorporation of acetate-1-C^{14} into lipids is significantly increased in both infarcted and uninfarcted areas of the injured muscle compared to normal heart muscle without coronary occlusion. Two days after infarction, the increase was greatest in the periphery of infarct, but 10 days after infarction, the increase in lipid synthesis in the center of the infarct exceeded that of the periphery.

The distribution of the incorporated acetate-1-C^{14} into the various lipid fractions is illustrated in Figure 8. The distribution of labeled acetate in the lipid fractions indicates a marked increase in triglyceride formation but a corresponding decrease in relative formation of phospholipids in the infarcted tissue compared to uninfarcted tissue of the same left ventricle.

The in vitro incorporation of acetate-1-C^{14} into lipids of a heart muscle homogenate as a function of the adenosine triphosphate concentration is illustrated in Figure 9. The rate of lipid synthesis was greatest at an ATP concentration of about 1 mM and diminished significantly at higher ATP concentrations.

The greatest increase was observed in the incorporation of acetate-1-C^{14} into triglycerides at a low ATP level, 1 mmole (mM). This low ATP level corresponds to the ATP content observed in infarcted heart muscle during the early period of tissue repair and development of fatty degeneration.

ENERGY METABOLISM

The state of aerobic and anaerobic energy metabolism was estimated by the determination of ATP and lactate in biopsy samples from normal and infarcted tissue obtained 2 days or 10 days after infarction. A relation between the tissue content of lactate and ATP was observed in infarcted tissue (Fig. 10). In normal heart muscle the ATP level was 5.57 ± 0.25 μmole(μM)/gm. and the lactate level was 1.72 ± 0.18 μM/gm. Two days after coronary occlusion, the ATP level had dropped to 0.88 ± 0.44 μM/gm., whereas the lactate concentration had increased to 5.76 ± 1.28 μM/gm. of infarcted tissue. Ten

days after infarction the ATP level had increased to 1.64 ± 0.21 μM/gm., but the lactate concentration diminished to 3.92 ± 0.66 μM/gm. (no. = 10). The inverse relation between glycolytic metabolism in infarcted tissue and the level of ATP is illustrated in Figure 11, which demonstrates a logarithmic increase in lactate formation as a function of diminishing ATP content.

Discussion

The metabolic alterations in an acutely ischemic heart muscle have been studied extensively, and a great deal has been learned about the regulation of myocardial glycolytic activity.[17] The studies of Chance et al.,[18] Williamson[19] and Wollenberger and Krause[17] indicate that the shift from aerobic to anaerobic metabolism in the myocardium following sudden coronary occlusion occurs in an oscillatory fashion. The principal control sites in the oscillating glycolytic system of anaerobic muscle have been identified as phosphorylase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase.[17]

In case of a permanent coronary occlusion the myocardial cells fail to survive the ischemia and, following a rapid depletion of energy-rich phosphates and glycogen, these cells die and disintegrate. The interpretation of the relative changes observed in the enzyme profile and metabolism of irreversibly damaged cardiac muscle cells is accompanied by numerous difficulties because the destruction of muscle cells and replacement of the necrotic muscle by connective scar tissue generates an ill defined, inhomogeneous tissue consisting of various cell types performing different tasks. The metabolism of the ischemic tissue, described in this study, can therefore not be ascribed to a specific cell population, but must be attributed to the collective effort of the various cell types involved in the repair and replacement of the damaged muscle.

The alterations in metabolism of infarcted heart muscle observed during tissue repair following sudden coronary occlusion are characterized by a significant increase in activity of the hexosemonophosphate (HMP)-shunt (Fig. 5) and by an increase in lipid synthesis (Table 1) paralleled by a rapid inactivation of oxidative and certain glycolytic enzymes (Fig. 2 to 4).

The diminution in energy level of infarcted tissue is illustrated by the decrease in ATP content of the ischemic tissue (Fig. 10). The inverse relation between the lactate level and the ATP level of

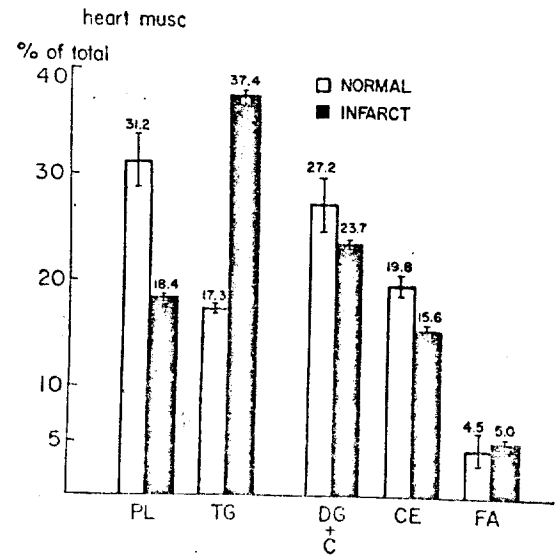


Figure 8. *Fractional distribution of acetate-1-C^{14} in lipids of normal and infarcted heart muscle* 24 hours after infarction. PL = phospholipids, TG = triglycerides, DG = diglycerides, CE = cholesterol-ester, FA = free fatty acids.

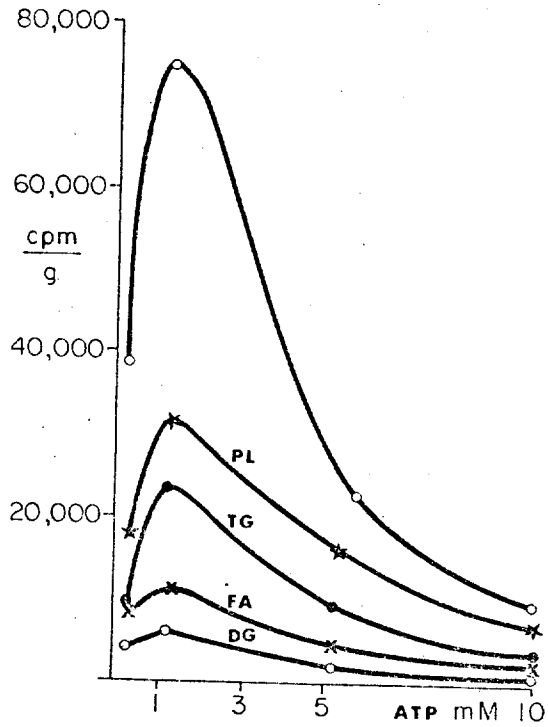


Figure 9. *The effect of ATP concentration upon the rate of incorporation of acetate-1-C^{14} into lipids of a heart muscle homogenate in vitro.* The top curve represents incorporation into total lipids; the other curves represent incorporation into the respective lipid fractions as a function of the ATP concentration of the medium. (Abbreviations as in Fig. 8.)

TABLE I
Incorporation of Acetate-1-C^{14} into Lipids of Infarcted Heart Muscle

Time After Coronary Occlusion (days)	μM of Acetate-1-C^{14} Incorporated into Lipids per Gram of Tissue			
	Normal LV (no. = 10)	Infarcted Heart Muscle		
		Uninfarcted Area of LV	Periphery of Infarct	Center of Infarct
	138 ± 10 (100%)			
2		383 ± 47 +177.5% No. = 4 p <0.01	488 ± 76 +253.6% No. = 4 p <0.01	336 ± 51 +143.5% No. = 4 p <0.01
10		302 ± 39 +118.8% No. = 6 p <0.01	497 ± 61.2 +260.1% No. = 6 p <0.01	586 ± 60 +325.4% No. = 6 p <0.01

LV = left ventricle.

the infarcted muscle indicates a significant rise in anaerobic metabolism despite the diminution in activity of glycolytic enzymes. The rise in ATP level and the diminution in lactate level of infarcted muscle, 10 days after infarction (Fig. 10), parallel the increase in HMP-shunt activity, but there was no increase in activity of mitochondrial enzymes (NAD-IDH) (Fig. 2).

The increased ATP formation 10 days after infarction can be explained by an increase in the rate of substrate phosphorylation at the level of GAPDH. The relatively high activity of GAPDH in infarcted tissue indicates that anaerobic phosphorylation of ADP to ATP can continue at a relatively high rate in the ischemic area, if the substrate glyceraldehyde-3-phosphate (GAP) can be provided. This could be accomplished in part by way of an increase in HMP-shunt activity during tissue repair, which would supplement the diminished output of the glycolytic pathway. The ischemic energy-deficient tissue might be able to increase its energy production by avoiding the ATP, requiring phosphorylation of fructose-6-phosphate (F-6-P) to fructose-1-6-diphosphate, directing instead the glucose-6-phosphate (G-6-P) into the HMP-shunt. Calculations of the quantitative role of the HMP-shunt in energy metabolism of infarcted tissue have not been attempted, but considerable recycling of F-6-P to G-6-P[20] must be assumed if this process could be expected to have any significance for the formation of GAP and ATP in the damaged tissue.

The possible role of GAPDH as a regulatory enzyme in the glycolytic metabolism of myocardial cells has been pointed out by Wollenberger.[17] This enzyme may have even a greater role in the energy production of infarcted tissue when the myocardial cells are replaced by more primitive connective tissue cells. The cells invading and multiplying in the severely ischemic area of the infarcted myocardium have to provide energy for numerous synthetic processes, among these protein and nucleic acid synthesis.[1]

The marked increase in ATP content of infarcted tissue 10 days after infarction occurs when oxidative and most glycolytic enzymes

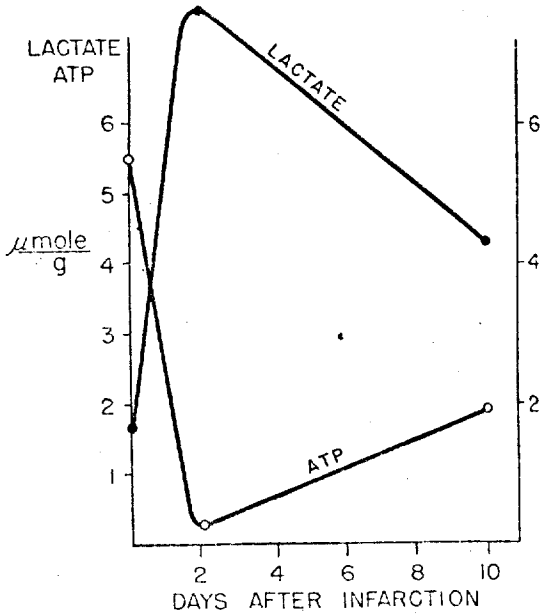


Figure 10. *The changes in ATP and lactate content of infarcted heart muscle* 2 days and 10 days after coronary occlusion. Each point represents the mean value of 6 to 10 determinations.

reach their lowest activity and when the enzymes of the HMP-shunt show a very significant increase in activity. The increase in HMP-shunt activity thus parallels an increase in ATP content and a decrease in lactate content of infarcted tissue. The decrease in lactate content may be associated with the increase in fatty acid formation in infarcted tissue, thereby reflecting an increase in the conversion of pyruvate to fatty acids (Fig. 12).

The increase in fatty acid and lipid formation seems to serve two functions: (1) to regenerate NADP required for continuous operation of the HMP-shunt and (2) to remove the product of the Embden-Meyerhof pathway, i.e., pyruvate via acetyl-CoA, and convert it into fatty acids and triglycerides. In this way, intermediary metabolites in fatty acid synthesis serve to substitute for oxygen and facilitate continued operation of both the HMP-shunt and the Embden-Meyerhof pathway in the ischemic tissue.

The role of the cellular energy level in the regulation of myocardial fatty acid and glyceride formation is illustrated in Figure 9, which shows that the rate of fatty acid synthesis is regulated by ATP. The rate of fatty acid synthesis was found to be stimulated by NADP or NADPH and low levels of ATP (1 to 2 mM), but it was markedly inhibited by physiologic concentration of ATP (5 to 6 mM). Glyceride formation was in turn regulated by the availability of activated fatty acids for esterification and, thus, was a function of the rate of fatty acid synthesis and cellular ATP content.[21] These observations indicate that the conditions most favorable to fatty acid and lipid formation in cardiac muscle are those existing in a hypoxic or ischemic muscle with diminished ATP level and increased level of NADH and NADPH.

The role of the HMP-shunt in metabolism of infarcted cardiac muscle is not limited to a possible effect on energy metabolism and lipid synthesis in the ischemic tissue; it also includes formation of ribose-5-phosphate required for nucleic acid synthesis, and formation of NADPH required for numerous biosynthetic processes. The increased rate of RNA synthesis has been studied by following the incorporation of glycine-2-C^{14} into the purine ring of RNA.[1] The earliest increase in incorporation was observed for "nucleolar RNA" four hours after infarction, followed by increased incorporation into nuclear ribosomal RNA. DNA showed an increase in incorporation 48 hours after infarction, and this increase coincided with the appearance of new fibroblasts.[1]

The increased HMP-shunt activity in the infarcted tissue may, however, not be due to tissue repair and fibroblastic proliferation alone. Dedifferentiation and degeneration of heart muscle cells may also play an important part in the increased HMP-shunt activity. Fujimoto and Harary[22] have observed significant enzyme changes associated with dedifferentiation and loss of function of heart muscle cells in tissue

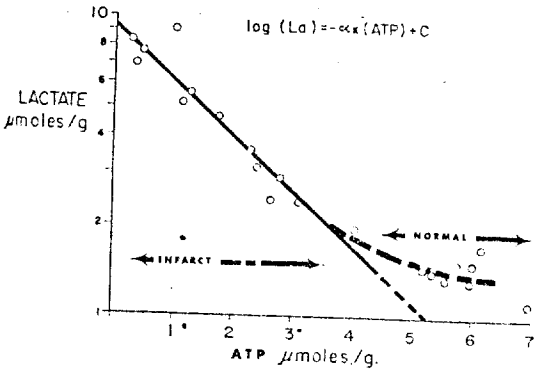


Figure 11. *An inverse relationship between ATP and lactate levels of infarcted heart muscle.* In normal heart muscle the ATP level was from 4.5 to 6.9 μM/gm. and the lactate level ranged from 1.3 to 1.9 μM/gm. In infarcted muscle there was a logarithmic increase in the lactate content as a function of the diminishing ATP level.

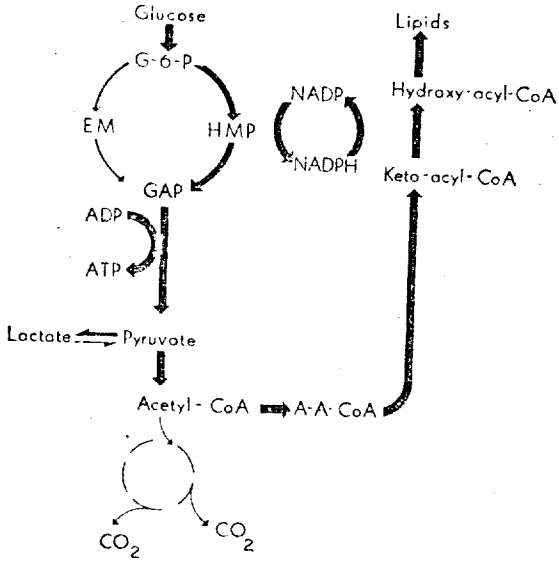


Figure 12. *Metabolism of infarcted heart muscle:* The heavy lines indicate metabolic pathways that have increased in activity in the infarcted tissue during the reparative processes [hexosemonophosphate-shunt (HMP) and lipid synthesis]. The thin lines indicate diminished activity of the Krebs cycle and the Embden-Meyerhof pathway.

culture. They observed a diminution of MDH and IDH activity but an increase in G-6-PDH activity when nondividing beating rat heart cells were grown in a lipid-free medium and subsequently ceased beating. The shift from lipid to carbohydrate oxidation and the loss of beating could be correlated with a decrease in oxygen uptake.

The increase in HMP-shunt activity of injured heart muscle is not limited to the ischemic area but also extends to uninfarcted areas of the left ventricle (Fig. 6), although to a much smaller degree. This increase in HMP-shunt activity of uninfarcted areas of injured muscle is accompanied by an increase in lipid synthesis of the uninfarcted tissue compared to normal heart muscle without injury (Table 1).

The major changes in the relative importance of the main metabolic pathways in infarcted heart muscle are characterized by diminished oxidative metabolism by way of the citric acid cycle, accompanied by quantitative alterations in activities of glycolytic enzymes (Fig. 12). The marked increase in HMP-shunt activity is paralleled by increased lipid synthesis and subsequent appearance of fatty degeneration in infarcted tissue. The HMP-shunt pathway provides substrates and cofactors for numerous anabolic reactions and stimulates nucleic acid and protein synthesis required for repair of the infarcted tissue.[1,23,24]

Summary

The metabolism of infarcted canine heart muscle was studied by examining the enzyme profile of infarcted and normal tissue; oxidative and glycolytic metabolism, as expressed in ATP and lactate levels of the infarcted muscle; and lipid synthesis and fatty degeneration of the infarcted muscle.

The activities of heart muscle enzymes were assayed during tissue repair following experimental myocardial infarction. The activities of 10 enzymes were determined in normal heart muscle and in the center and periphery of infarcted muscle. A significant increase in hexosemonophosphate (HMP)-shunt activity was observed in infarcted tissue 5 hours after infarction. The activities of glucose-6-phosphate dehydrogenase (G-6-PDH) and 6-phosphogluconate dehydrogenase (6-PGDH) increase 31–34-fold 10 days after infarction. In contrast to increased activity of the HMP-shunt, there was a rapid decline in oxidative and glycolytic enzyme activities in infarcted tissue. Oxidative enzymes reached the lowest level in center of infarcted tissue 10 days after infarction with 8 to 13 per cent of control activities. Glycolytic enzymes showed a significant fall in activities with the greatest diminution in activity of aldolase to 14 per cent of control but a relatively smaller fall in glyceraldehyde phosphate dehydrogenase (GAPDH) activity. The increased activity of the HMP-shunt in infarcted tissue was also expressed in the relative oxidation of glucose-1-C^{14} and glucose-6-C^{14} to $C^{14}{}_{O_2}$.

The diminution in oxidative metabolism was also expressed in a marked fall (85 to 90 per cent decrease) in adenosine triphosphate (ATP) content of the infarcted tissue, and a significant rise in lactate content two days after infarction. Ten days after coronary occlusion the cellular ATP level increased again with a parallel decrease in lactate content.

The incorporation of acetate-1-C^{14} into lipids was significantly increased in both infarcted and uninfarcted areas of the injured muscle, compared to normal heart muscle without coronary occlusion. The distribution of labeled acetate in the lipid fractions indicated a marked increase in triglyceride formation in the infarcted tissue.

The rate of fatty acid and glyceride synthesis was found to be regulated by the ATP level, stimulated at low ATP levels (1 to 2 mM) but markedly inhibited at physiological ATP levels (5 to 6 mM). The conditions most favorable for lipid synthesis in cardiac muscle are those of a hypoxic or ischemic muscle with diminished ATP level.

References

1. Gudbjarnason, S., DeSchryver, C., Chiba, C., Yamanaka, J. and Bing, R. J. Protein and nucleic acid synthesis during the reparative process following myocardial infarction. *Circulation Res.*, 15: 320, 1964.
2. Goebell, H. and Klingenberg, M. DPN-spezifische Isocitrat-Dehydrogenase der Mitochondrien. 1. Kinetische Eigenschaften, Vorkomen und Funktion dem DPN spezifische Isocitrat-Dehydrogenase. *Biochem. Ztrschr.*, 340: 441, 1964.
3. Bücher, T. and Klingenberg, M. Wege des Wasserstoffs in der lebendigen Organisation. *Angew. Chemie.*, 70: 552, 1958.
4. Schimasek, H. Enzymatische Analyse der Glatten Muskulatur in Vergleich zu anderen muskelgeweben unter dem Aspekt der Embryologischen Zuordnung. *Biochem. Ztschr.*, 333: 463, 1961.
5. Delbrück, A., Zebe, E. and Bücher, T. Über Verteilungsmuster von Enzymen des Energieliefernden Stoffwechsels im Flugmuskel, Sprungmuskel und Fettkörper von Locusta Migratoria und ihre cytologische Zuordnung. *Biochem. Ztschr.*, 331: 273, 1959.

6. Bücher, J., Luh, W. and Pette, D. Einfache und Zusammengesetzte optische Tests mit Pyridinnucleotiden. Handbuch der Physiologisch und Pathologisch-Chemischen Analyse, Vol. 6A, p. 292, 1964. Berlin, Hoppe-Seyler/Thierfelder.

7. Horecker, B. L. and Kornberg, A. The extinction coefficient of the reduced band of pyridine nucleotides. *J. Biol. Chem.*, 175: 385, 1948.

8. Henry, R. J., Sobel, C. and Berkman, S. Interferences with Biuret methods for serum proteins. *Anal. Chem.*, 29: 1491, 1957.

9. Folch, J., Lees, M. and Sloan-Stanley, G. J. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biochem.*, 226: 497, 1957.

10. Freeman, C. P. and West, D. Complete separation of lipid classes on a single thin-layer plate. *J. Lipid Res.*, 7: 324, 1966.

11. Hohorst, H. T. L-(+)-lactate. Determination with lactic dehydrogenase and DPN. In: Methods of Enzymatic Analysis, p. 266. Edited by Bergmeyer, H. U. New York and London, 1963. Academic Press.

12. Adam, H. Adenosine-5-triphosphate. Determination with phosphoglycerate kinase. In Ref. 11, p. 539.

13. Katz, J. and Wood, H. G. The use of $^{14}CO_2$ yields from glucose-1-C^{14} and 6-C^{14} for the evaluation of the pathways of glucose metabolism. *J. Biol. Chem.*, 238: 517, 1963.

14. Crevasse, L. E., Shipp, T. C. and Delcher, H. K. Glucose metabolism by the hexose monophosphate pathway in heart muscle homogenates. *Biochim. et biophys. acta.*, 86: 402, 1964.

15. Shipp, J. D., Delcher, H. K. and Crevasse, L. E. Glucose metabolism by the hexose monophosphate pathway in the perfused rat heart. *Biochim. et biophys. acta*, 86: 399, 1964.

16. Hoskin, F. C. G. A source of error in the use of radioactive substrates for metabolic studies. *Arch. Biochem.*, 87: 151, 1960.

17. Wollenberger, A. and Krause, E. G. Metabolic control characteristics of the acutely ischemic myocardium. *Am. J. Cardiol.*, 22: 349, 1968.

18. Chance, B., Williamson, J. R., Jamieson, D. and Schoener, B. Properties and kinetics of reduced pyridine nucleotide fluorescence of the isolated and in vivo rat heart. *Biochem. Ztschr.*, 341: 357, 1965.

19. Williamson, J. R. Glycolytic control mechanism. II. Kinetics of intermediate changes during the aerobic-anoxic transition in perfused rat heart. *J. Biol. Chem.*, 241: 5026, 1966.

20. Wood, H. G., Katz, T. and Landau, B. R. Estimation of pathways of carbohydrate metabolism. *Biochem. Z.*, 338: 809, 1963.

21. Gudbjarnason, S. Regulation of glyceride biosynthesis in heart muscle. *Fed. Proc.*, 26/2: 658, 1967.

22. Fujimoto, A. and Harary, J. The effect of lipids on enzyme levels in beating rat heart cells. *Biochem. Biophys. Res. Commun.*, 20: 456, 1965.

23. Gudbjarnason, S. and DeSchryver, C. Nuclear ribosomes. An early factor in tissue reparation. *Biochem. Biophys. Res. Commun.*, 14: 12, 1964.

24. Gudbjarnason, S., Fenton, J., Wolf, P. L. and Bing, R. J. Stimulation of reparative processes following experimental myocardial infarction. *Arch. Int. Med.*, 118: 33, 1966.

German Translation

Effect of Volatile Acids in Saliva on the Aerobic Metabolism of Oral Bacteria*

B. Guggenheim, L. Ettlinger and H.R. Muhlemann (From the Caries Research Station at the Dental Institute of Zurich University and the Microbiology Institute of Zurich Polytechnic Institute)

Source: Pathologia et Microbiologia 28, No. 1: 77-83 (1965)

In 1955, Hartles and Wasdell noted the presence, in the supernatant of centrifuged saliva, of a heat-resistant and dialyzable factor which activated the respiration and glycolysis of oral bacteria. This factor could not consist of an anorganic ion, since its effect was suppressed by calcination. In 1957, the factor was again discovered by Bramstedt et al., and its existence was confirmed in 1961 by Beck and Bibby. With the exception of the study of Beck and Bibby, who used the periodic titration of acid end-products as a measuring scale for the activation, the other studies were carried out by the usual Warburg methods. Both Hartles and Wasdell and Beck and Bibby used a washed sediment of stimulated mixed saliva as the source of microorganisms, whereas Bramstedt et al. used a pure culture of Candida albicans.

In the present study, mixed saliva stimulated with plastic chewing tablets was obtained from approximately 240 school children. The centrifuged saliva (10 min at 10,000 g and 4°C) was processed in an autoclave for 20 min at 120°C, the supernatant was then centrifuged once more and

* These studies were supported by a grant (DE-1436) from the U.S. Public Health Service, National Institutes of Health Bethesda, Md. and by a research grant from the Swiss Dental Association.

processed in an autoclave, in order to remove a colloidal turbidity probably formed by denaturation of heat-sensitive macromolecules. The sediment was discarded. Out of a total of 24 strains isolated from salivary sediment, that strain was used further as test organism the respiration of which in the Warburg apparatus was activated the most by addition of saliva supernatant. This strain was a gram-positive, aerobic organism of the Micrococcaceae family, which does not belong to any of the species described in Bergey's manual (7th edition). For the Warburg tests, the cells were incubated in broth at 37°C for 14 hours, then centrifuged twice and washed. The sediment was collected (absorbed) in a phosphate buffer (pH 6.8) and homogenized with the aid of a magnetic stirrer. The density of the suspension was adjusted by nephelometry, so that 1 ml had an average dry substance content of 6.89 mg with a standard deviation of $\pm$ 0.22 mg (N = 27). The adapter used in the exogenous Warburg tests had a phosphate concentration of 1/15 M and a glucose concentration of 0.005 M. Each small flask equipped with two adapter vessels was filled with 1 ml test solution, 0.5 ml glucose solution, 1 ml bacterial suspension and 0.2 ml 20% KOH solution. Within a Warburg test, every measurement was carried out with 3 parallel determinations (one group). Along with a control group, the test included one to three test groups. The average oxygen absorption (in µl O_2) was calculated for each group. The duration of the test was limited to exactly 1 hour. On the basis of this arrangement, the activity was defined as the difference between the average O_2-absorption of the groups with the test solution and the control group. One activity unit therefore corresponds to the additional absorption (uptake) of 1 µl O_2 per ml test solution per hour in comparison to the control.

After compiling the activity balance and checking eluates of paper chromatograms with acetic acid as a component of the solvent, it was assumed that traces of acetic acid could exert an activating effect. Consequently, the content of volatile acids in the saliva was investigated. From 200 ml supernatant, a total of 700-800 ml distillate was fractionally recovered by steam distillation. Aliquot portions were titrated exactly with 0.010 N NaOH to a pH of 7.00, and the activity of individual fractions was checked. As can be seen from Table I Fig. 1, the activity of the fractions depends on the content of volatile acids. In order to detect any eventual activity losses during distillation, balances were compiled. Table II shows that at least 60% of the total activity can be attributed to volatile acids. Distillation did not result in any activity losses. The nature of the volatile acids was determined by gas chromatography (Fig. 2). This method, together with total acid determination, also allowed us to make a quantitative estimate (evaluation) of the results (Table III). The activity of pure acids was investigated. In addition to volatile acids, lactic acid was tested, since this acid is the one most frequently mentioned in connection with dental caries. As can be seen from Table IV, all these acids have an activating effect, with the exception of Na formate. A colorimetric lactic acid determination according to Barker and Summerson showed that surprisingly small amounts of lactic acid are present in saliva supernatant (Table V). In view of the considerable amounts of volatile acids, and primarily of acetate and propionate, we had reason to assume that a substrate effect, rather than an activation proper, takes place. This hypothesis was investigated by testing (checking) glucose as a substrate and acids as presumptive substrates in a 2^2-factorial order

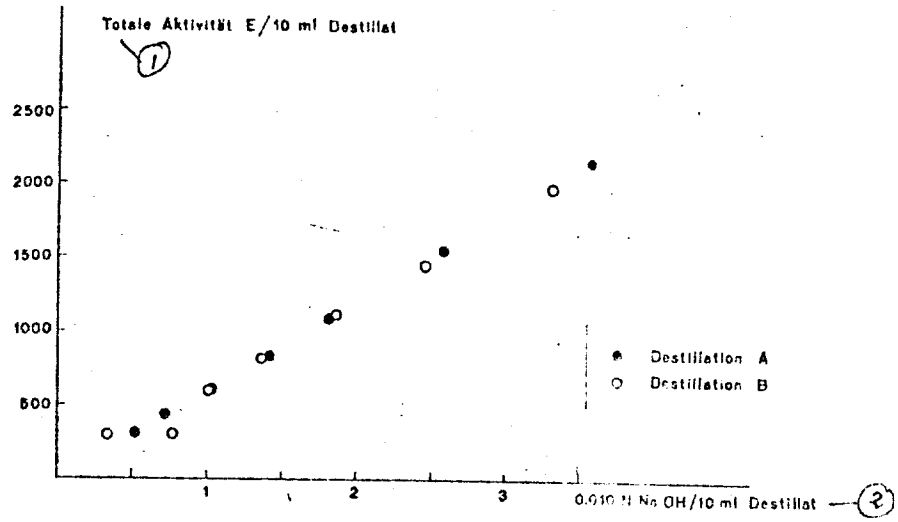
(Table VI). As Table VII shows, there is no significant mutual effect between glucose and the various acids. Thus, individual components merely exert an additive effect, and an activation proper (in the actual sense of the word) is absent.

Table I

Titratable Acid And Activity of Fractions Obtained From Two Steam Distillations (A, B) of Mixed Saliva (Two Titrations)

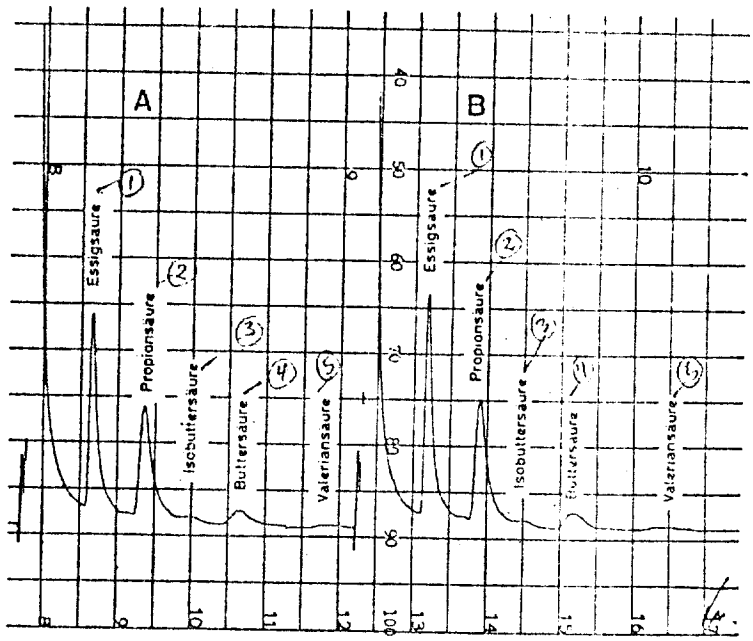
(1) Fraktionen à 100 ml No.		(2) ml 0.010 N NaOH pro 10 ml Destillat		(3) Aktivität E.
A.	1	3,60	3,56	214,8
	2	2,58	2,59	155,1
	3	1,82	1,85	110,1
	4	1,40	1,40	84,0
	5	1,05	1,03	62,4
	6	0,73	0,73	43,8
	7	0,51	0,54	31,5
B.	1	3,33	3,27	198,0
	2	2,46	2,47	147,9
	3	1,84	1,89	111,9
	4	1,37	1,37	82,3
	5	1,03	1,02	61,5
	6	0,77	0,77	46,2
	7	0,52	0,51	30,9
	8	0,34	0,33	30,1

1 -- 100 ml fractions-No.
2 -- ml of 0.010 N NaOH per 10 ml distillate
3 -- Activity, units



1 -- Total activity E/10 ml distillate.
2 -- 0.010 N NaOH/10 ml distillate

Fig. 1. Total activity of fractions obtained from two steam distillations (A, B) as a function of titratable acid.



1 -- Acetic
2 -- Propionic acid
3 -- Isobutyric acid
4 -- Butyric acid
5 -- Valeric acid

Fig. 2. Gas chromatograms of steam distillates (A, B) of saliva supernatant.

Table II

Activity Balance After One Steam Distillation

	Art der Probe	Volumen ml	Aktivität		Total Aktivität	
			L.	%	L.	%
Saliva →	Speichel	200	253,7		50 740	100,0
Fraction	Fraktion 1	100	117,7		11 770	23,2
	Fraktion 2	100	69,7		6 970	13,7
	Fraktion 3	100	50,9		5 090	10,2
	Fraktion 4	100	37,6		3 760	7,4
	Fraktion 5	100	28,8		2 880	5,7
	Fraktion 6	100	25,1		2 510	4,9
	Fraktion 7	100	18,6		1 860	3,7
	Fraktion 8	100	10,4		1 040	1,8
Fractions →	Fraktionen 1–8	800			35 880	70,7
Residue →	Rückstand	200	88,0		17 600	34,7
					53 480	105,4
Saliva →	Speichel	1	253,7	100,0		
Combined total distillate →	Zusammengesetztes Gesamtdestillat	1	154,4	60,9		
Residue →	Rückstand	1	88,0	34,7		
			242,4	95,6		
Residue and combined total distillate	Rückstand und zusammengesetztes Gesamtdestillat	1	260,2	102,6		

1 -- Type of sample
2 -- Activity
3 -- Total activity

Table III

Quantitative Evaluation of Gas Chromatograms (see Fig. 2)

(1) Art der Säure		(2) Fläche %	mg/l	mMol
A.	Essigsäure (3)	48	198,5	3,31
	Propionsäure (4)	39	161,3	2,18
	Isobuttersäure (5)	3	12,4	0,14
	Buttersäure (6)	7	28,9	0,33
	Valeriansäure (7)	3	12,4	0,12
	Total	100	413,6	6,08
B.	Essigsäure (3)	47	191,9	3,20
	Propionsäure (4)	41	167,4	2,26
	Isobuttersäure (5)	3	12,3	0,14
	Buttersäure (6)	6	24,5	0,28
	Valeriansäure (7)	3	12,3	0,12
	Total	100	408,4	6,00

1 -- Type of acid
2 -- Area, %
3 -- Acetic acid
4 -- Propionic acid
5 -- Isobutyric acid
6 -- Butyric acid
7 -- Valeric acid

Table IV

Activities of Lactic Acid And Some Volatile Acids

(1) Konzentration berechnet für freie Säuren mMol	(2) Na-Formiat Aktivität E	(3) Essigsäure Aktivität E	(4) Na-Acetat Aktivität E	(5) Milchsäure Aktivität E	(6) Na-Propionat Aktivität E
1	10,5	39,6	37,3	27,9	60,0
2	0	52,7	54,5	55,2	104,3
3	2,5	81,6	85,8	88,5	146,8
4	8,1	105,4	111,8	106,2	155,7

1 -- Concentration, Calculated for free acids, in mMoles
2 -- Na formate, activity units
3 -- Acetic acid, activity units
4 -- Na acetate, activity units
5 -- Lactic acid, activity units
6 -- Na propionate, activity units

Table V

Lactic Acid Activity in Mixed Saliva, Combined Total Distillate And Distillation Residue

	Art der Probe	(4) Milchsäure mg/l			Durchschnitt (5)
(1)	Speichel	21,7	22,2	25,2	23,0
(2)	Zusammengesetztes Gesamtdestillat	0	0	0,5	≤ 0,5
(3)	Destillationsrückstand	11,9	11,9	11,1	11,6

1 -- Saliva
2 -- Combined total distillate
3 -- Distillation residue
4 -- Lactic acid, mg/l
5 -- Average

Table VI

Average O_2-Absorption (µl/hr) With Different Substrates (N=6)

	Faktor a – Factor a	o	a	g	ag
(1)	Essigsäure 180 mg/l	113,5	178,9	181,1	242,6
(2)	Na Propionat 230 mg/l	113,8	228,2	176,4	242,5
(3)	Milchsäure 180 mg/l	109,0	165,2	161,3	217,5
(4)	Essigsäure und Na-Propionat	109,0	258,5	157,7	301,5

1 -- Acetic acid, 180 mg/l
2 -- Na propionate, 230 mg/l
3 -- Lactic acid, 180 mg/l
4 -- Acetic acid and Na propionate

o - endogenous conversion; a - volatile acid; g - glucose; ag - volatile acid + glucose

Table VII

Average Effect of Glucose And Different Acids on Oxygen Absorption (µl O_2/hr)

	Glucose	+ 65,7		Glucose	+ 52,3
1	Essigsäure	+ 63,4	4	Milchsäure	+ 56,2
2	Wechselwirkung	— 0,2	2	Wechselwirkung	— 0,4
	Glucose	+ 63,4		Glucose	+ 45,9
3	Na-Propionat	+ 115,2	5	Essigsäure und Na-Proprionat	+ 146,6
2	Wechselwirkung	— 0,8	2	Wechselwirkung	— 2,8

1 -- Acetic acid 2 -- Mutual effect
3 -- Na propionate 4 -- Lactic acid
5 -- Acetic acid and Na propionate

The low latic acid content in stimulated mixed saliva, as compared with the content of volatile acids, allows us to assume that lactic acid is converted into volatile acids. Whether this conversion can take place after stimulation in the bacterial-saliva mixture, namely prior to centrifugation and autoclaving, or in situ (dental plaque), remains open. The breakdown of acids by bacterial respiration is a question of basic interest in regard to dental caries.

Summary

Supernatant of stimulated, mixed and pooled saliva of 240 school children was used for an investigation of the activating factor described by Hartles and Wasdell in 1955. This factor was believed to enhance aerobic and anaerobic activity of salivary sediment. A test method developed for this purpose, using conventional Warburg techniques, and a pure strain of an oral bacterium were used to test the activating properties of fractions prepared from supernatant. 60% of its total acti-

vity could be attributed to 5 volatile acids, which were identified by gas chromatography. The results indicate that the effect, previously supposed to be an activation, is due in part to metabolic breakdown of the volatile acids. They are assumed to be formed by certain oral strains, and are available in saliva, as a substrate for some oral microorganisms.

Bibliography

1. Barder, S.B. and Summerson, W.H.: The colorometric determination of lactic acid in biological material. J. biol. Chem. 138:535 (1941).

2. Beck, D.J. and Bibby, B.G.: Whole saliva, salivary sediment and supernatant as fermenting agents for food. J. Dent. Res. 40:479 (1961).

3. Bramstedt F., Kroncke A. and Naujoks R.: On a factor in human saliva which activates anaerobic carbohydrate metabolism, Naturwissenschaften 44:331 (1957).

4. Hartles, R.L. and Wasdell, Marie R.: The metabolism of the oral flora. VI. Preliminary observations on a water-soluble factor in saliva which enhances the respiratory and glycolytic activity of the salivary flora. Brit. Dent. J. 98:334 (1955).

The authors with to express their gratitude to Dr. A.L. Prabucki, of the Zurich Polytechnic Institute, for carrying out the gas-chromatographic analysis.

23. Jahresvers. Schweiz. Mikrobiol. Ges., Genf 1964
Path. Microbiol. *28:* 77–83 (1965)

Kariesforschungsstation, Zahnärztliches Institut der Universität Zürich, und Institut für Mikrobiologie an der Eidgenössischen Technischen Hochschule Zürich

Die Wirkung von flüchtigen Säuren im Speichel auf den aeroben Metabolismus von Mundbakterien*

Von B. GUGGENHEIM, L. ETTLINGER und H. R. MÜHLEMANN

Hartles und Wasdell beobachteten 1955 im Überstand von zentrifugiertem Speichel einen hitzeresistenten und dialysierbaren Faktor, der die Atmung und Glykolyse von Mundbakterien aktivierte. Bei diesem Faktor konnte es sich nicht um ein anorganisches Ion handeln, da ein Veraschen die Wirkung zum Verschwinden brachte. 1957 wurde der Faktor von *Bramstedt* et al. wiederentdeckt, 1961 seine Existenz von *Beck und Bibby* bestätigt. Mit Ausnahme der Arbeit von *Beck und Bibby*, die die periodische Titration der sauren Endprodukte der Testbakterien als Gradmesser für die Aktivierung benutzten, wurden die anderen Arbeiten mit konventionellen Warburg-Methoden durchgeführt. Sowohl *Hartles und Wasdell* als auch *Beck und Bibby* verwendeten das gewaschene Sediment von stimuliertem Mischspeichel als Mikroorganismenquelle, währenddem *Bramstedt* et al. eine Reinkultur von *Candida albicans* benutzten.

Für die vorliegende Arbeit wurde von ungefähr 240 Schulkindern mit Kunststoffkautabletten stimulierter Mischspeichel gewonnen. Der zentrifugierte Speichel (10 min, 10000 g, 4° C) wurde autoklaviert (20 min, 120° C), der Überstand anschließend nochmals zentrifugiert und autoklaviert, um eine kolloidale Trübung, vermutlich entstanden durch eine Denaturierung hitzelabiler Makromoleküle, zu entfernen. Das Sediment wurde verworfen. Von

* Diese Untersuchungen wurden unterstützt durch einen Beitrag (DE-1436) des U.S. Public Health Service, National Institutes of Health, Bethesda, Md., sowie durch einen Forschungsbeitrag der Schweizerischen Zahnärztegesellschaft.

TABELLE 1 Tabelle I

Titrierbare Säure und Aktivität von Fraktionen erhalten aus zwei Wasserdampfdestillationen (A, B) von gemischtem Speichel (zwei Titrationen)

(1) Fraktionen à 100 ml No.	(2) ml 0.010 N NaOH pro 10 ml Destillat		(3) Aktivität E.
A. 1	3,60	3.56	214,8
2	2,58	2,59	155,1
3	1,82	1,85	110,1
4	1,40	1,40	84,0
5	1,05	1,03	62,4
6	0,73	0,73	43,8
7	0,51	0,54	31,5
B. 1	3,33	3,27	198,0
2	2,46	2,47	147,9
3	1,84	1,89	111,9
4	1,37	1,37	82,3
5	1,03	1,02	61,5
6	0,77	0,77	46,2
7	0,52	0,51	30,9
8	0,34	0,33	30,1

insgesamt 24 Stämmen, die aus Speichelsediment isoliert wurden, fand derjenige Stamm als Testorganismus weitere Verwendung, dessen Atmung im Warburg-Apparat durch die Zugabe von Speichelüberstand am meisten aktiviert wurde. Es handelte sich dabei um einen Gram-positiven, aeroben Organismus der Familie *Micrococcaceae*, der zu keiner der in *Bergey* (7. Auflage) beschriebenen Arten paßt. Für die Warburg-Versuche wurden die Zellen in Bouillon bei 37° C 14 Stunden inkubiert, danach zweimal zentrifugiert und gewaschen. Das Sediment wurde in Phosphatpuffer (pH 6,8) aufgenommen und mittels eines Magnetrührers homogenisiert. Die Dichte der Suspension wurde nephelometrisch eingestellt, so daß 1 ml einen durchschnittlichen Trockensubstanzgehalt von 6,89 mg bei einer Standardabweichung von ± 0,22 mg enthielt (N = 27). Im Ansatz für die exogenen Warburg-Versuche war die Phosphatkonzentration 1/15 M und die Glukosekonzentration 0.005 M. Pro Kölbchen mit zwei Ansatzbirnen wurden 1,0 ml Testlösung, 0,5 ml Glukoselösung, 1,0 ml Bakteriensuspension und 0,2 ml KOH 20% eingefüllt. Innerhalb eines Warburg-Versuchs erfolgte jede Messung mit drei Parallelen (eine Gruppe).

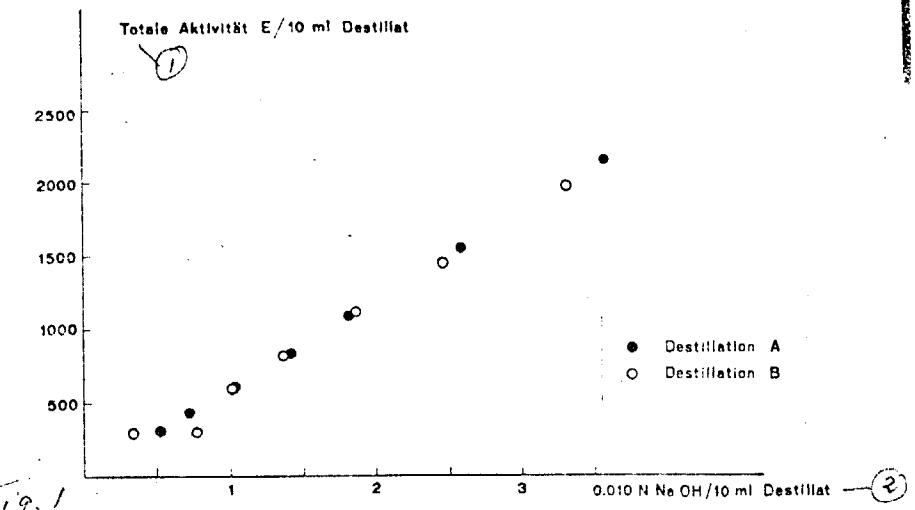


Fig. 1

Abb. 1. Totale Aktivität von Fraktionen erhalten aus zwei Wasserdampfdestillationen (A, B) in Funktion der titrierbaren Säure.

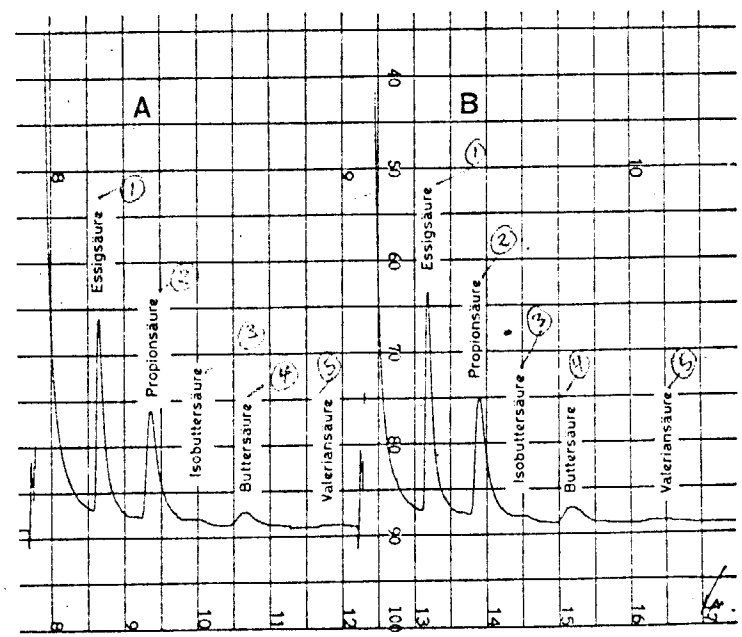


Abb. 2. Gaschromatogramme von Wasserdampfdestillaten (A, B) von Speichelüberstand.

Fig. 2

Neben einer Kontrollgruppe befanden sich eine bis drei Testgruppen im Versuch. Die durchschnittliche Sauerstoffaufnahme (μl O_2) wurde für jede Gruppe berechnet. Die Versuchsdauer wurde auf genau eine Stunde beschränkt. Auf Grund dieser Anordnung wurde die Aktivität als Differenz der durchschnittlichen O_2-Aufnahme zwischen den Gruppen mit Testlösung und der Kontrollgruppe definiert. Eine Aktivitätseinheit entspricht demnach der zusätzlichen Aufnahme von einem μl O_2 pro ml Testlösung und Stunde im Vergleich zur Kontrolle.

Daß Spuren von Essigsäure aktivieren, wurde nach der Aufstellung einer Aktivitätsbilanz, nach der Prüfung von Eluaten von Papierchromatogrammen mit Essigsäure als Komponente des Lösungsmittels, vermutet. In der Folge wurde daher der Gehalt an flüchtigen Säuren im Speichel untersucht. Von 200 ml Überstand wurden in Wasserdampfdestillationen 700—800 ml Destillat fraktioniert aufgefangen. Aliquote Teile wurden mit 0,010 N NaOH genau auf pH 7,00 titriert. Die Aktivität der einzelnen Fraktionen wurde geprüft. Wie Tab. I und Abb. 1 zeigen, ist die Aktivität der Fraktionen vom Gehalt an flüchtigen Säuren abhängig. Um eventuelle Aktivitätsverluste durch die Destillation zu entdecken, wurden Bilanzen erstellt. Aus Tab. II ist ersichtlich, daß mindestens 60% der Gesamtaktivität auf flüchtige Säuren zurückführbar ist. Die Destillation hatte keine Aktivitätsverluste zur Folge. Die Art der flüchtigen Säuren wurde durch Gas-Chromatographie bestimmt (Abb. 2). Dieses Verfahren zusammen mit der Gesamtsäurebestimmung erlaubte auch eine quantitative Auswertung der Resultate (Tab. III). Die Aktivität reiner Substanzen wurde untersucht. Zusätzlich zu den flüchtigen Säuren wurde auch noch Milchsäure, die im Zusammenhang mit Zahnkaries meistgenannte Säure, getestet. Wie aus Tab. IV hervorgeht, aktivieren alle diese Säuren mit Ausnahme von Formiat. Eine kolorimetrische Milchsäurebestimmung nach *Barker und Summerson* zeigte erstaunlich geringe Mengen von Milchsäure im Speichelüberstand (Tab. V). In Anbetracht der beträchtlichen Mengen von flüchtigen Säuren, vor allem Acetat und Propionat, war es gegeben, nicht eine eigentliche Aktivierung, sondern einen Substrateffekt zu vermuten. Diese Hypothese wurde durch Prüfung von Glukose als Substrat und von Säuren als präsumtiven Substraten in 2^2-faktorieller Anordnung untersucht (Tab. VI). Wie Tab. VII zeigt, ist keine signifikante Wechselwirkung zwischen Glukose und den

TABELLE II Table II

Aktivitätsbilanz nach einer Wasserdampfdestillation

(1) Art der Probe	Volumen ml	(2) Aktivität E.	Aktivität %	Totale Aktivität E.	Totale Aktivität % (3)
Saliva → Speichel	200	253,7		50 740	100,0
Fraktion 1	100	117,7		11 770	23,2
Fraktion 2	100	69,7		6 970	13,7
Fraktion 3	100	50,9		5 090	10,2
Fraktion 4	100	37,6		3 760	7,4
Fraktion 5	100	28,8		2 880	5,7
Fraktion 6	100	25,1		2 510	4,9
Fraktion 7	100	18,6		1 860	3,7
Fraktion 8	100	10,4		1 040	1,8
Fractions → Fraktionen 1–8	800			35 880	70,7
Residue → Rückstand	200	88,0		17 600	34,7
				53 480	105,4
Saliva → Speichel	1	253,7	100,0		
Combined total distillate → Zusammengesetztes Gesamtdestillat	1	154,4	60,9		
Residue → Rückstand	1	88,0	34,7		
		242,4	95,6		
Residue and combined total distillate → Rückstand und zusammengesetztes Gesamtdestillat	1	260,2	102,6		

TABELLE III Table III

Quantitative Auswertung der Gaschromatogramme (siehe Abb. 2)

(1)	Art der Säure	(2) Fläche %	mg/l	mMol
A.	Essigsäure (3)	48	198,5	3,31
	Propionsäure (4)	39	161,3	2,18
	Isobuttersäure (5)	3	12,4	0,14
	Buttersäure (6)	7	28,9	0,33
	Valeriansäure (7)	3	12,4	0,12
	Total	100	413,6	6,08
B.	Essigsäure (3)	47	191,9	3,20
	Propionsäure (4)	41	167,4	2,26
	Isobuttersäure (5)	3	12,3	0,14
	Buttersäure (6)	6	24,5	0,28
	Valeriansäure (7)	3	12,3	0,12
	Total	100	408,4	6,00

305

TABELLE IV Tabelle IV

Aktivitäten von Milchsäure und einigen flüchtigen Säuren

Konzentration berechnet für freie Säuren mMol	Na-Formiat Aktivität E	Essigsäure Aktivität E	Na-Acetat Aktivität E	Milchsäure Aktivität E	Na-Propionat Aktivität E
1	10,5	39,6	37,3	27,9	60,0
2	0	52,7	54,5	55,2	104,3
3	2,5	81,6	85,8	88,5	146,8
4	8,1	105,4	111,8	106,2	155,7

TABELLE V Tabelle V

Gehalt an Milchsäure in gemischtem Speichel, zusammengesetztem Gesamtdestillat und Destillationsrückstand

Art der Probe	Milchsäure mg/l			Durchschnitt
Speichel	21,7	22,2	25,2	23,0
Zusammengesetztes Gesamtdestillat	0	0	0,5	$\leq 0,5$
Destillationsrückstand	11,9	11,9	11,1	11,6

TABELLE VI Tabelle VI

Durchschnittliche O_2-Aufnahme (µl/h) mit verschiedenen Substraten ($N = 6$)

Faktor a	o	a	g	ag
Essigsäure 180 mg/l	113,5	178,9	181,1	242,6
Na-Propionat 230 mg/l	113,8	228,2	176,4	242,5
Milchsäure 180 mg/l	109,0	165,2	161,3	217,5
Essigsäure und Na-Propionat	109,0	258,5	157,7	301,5

o: endogener Umsatz; a: flüchtige Säure; g: Glucose; ag: flüchtige Säure + Glucose.

TABELLE VII Tabelle VII

Mittlere Wirkung von Glucose und verschiedenen Säuren auf die Sauerstoffaufnahme (µl O_2/h)

Glucose	+ 65,7	Glucose	+ 52,3
Essigsäure	+ 63,4	Milchsäure	+ 56,2
Wechselwirkung	— 0,2	Wechselwirkung	— 0,4
Glucose	+ 63,4	Glucose	+ 45,9
Na-Propionat	+ 115,2	Essigsäure und Na-Proprionat	+ 146,6
Wechselwirkung	— 0,8	Wechselwirkung	— 2,8

verschiedenen Säuren vorhanden. Die einzelnen Komponenten haben also bloß additive Wirkung, eine Aktivierung im eigentlichen Sinne fehlt.

Der geringe Gehalt an Milchsäure in stimuliertem Mischspeichel, verglichen mit dem Gehalt an flüchtigen Säuren, läßt eine Umwandlung von Milchsäure in flüchtige Säuren vermuten. Ob diese Umwandlung nach der Stimulation im Bakterien-Speichelgemisch, also vor dem Zentrifugieren und Autoklavieren, oder *in situ* (Zahnplaque) geschehen kann, bleibt offen. In bezug auf die Zahnkaries ist der Abbau von Säuren durch Bakterienatmung von grundlegendem Interesse.

Summary

Supernatant of stimulated, mixed and pooled saliva of 240 school children was used for an investigation of the activating factor described by *Hartles and Wasdell* in 1955. This factor was believed to enhance aerobic and anaerobic activity of salivary sediment. A test method developed for this purpose, using conventional Warburg techniques, and a pure strain of an oral bacterium were used to test the activating properties of fractions prepared from supernatant. 60% of its total activity could be attributed to 5 volatile acids, which were identified by gas chromatography. The results indicate that the effect, previously supposed to be an activation, is due in part to metabolic breakdown of the volatile acids. They are assumed to be formed by certain oral strains, and are available in saliva, as a substrate for some oral microorganisms.

Literatur

1. *Barker, S. B.* and *Summerson, W. H.:* The colorometric determination of lactic acid in biological material. J. biol. Chem. *138:* 535 (1941).
2. *Beck, D. J.* and *Bibby, B. G.:* Whole saliva, salivary sediment and supernatant as fermenting agents for food. J. dent. Res. *40:* 479 (1961).
3. *Bramstedt, F.; Kröncke, A.* und *Naujoks, R.:* Über ein den anaeroben Kohlenhydratabbau aktivierendes Prinzip im menschlichen Speichel. Naturwissenschaften *44:* 331 (1957).
4. *Hartles, R. L.* and *Wasdell, Marie R.:* The metabolism of the oral flora. VI. Preliminary observations on a water-soluble factor in saliva which enhances the respiratory and glycolytic activity of the salivary flora. Brit. dent. J. *98:* 334 (1955).

Für die Ausführung der gas-chromatographischen Analyse sind die Autoren Herrn Dr. A. L. Prabucki (ETH) zu Dank verpflichtet.

305

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 242, No. 16, Issue of August 25, pp. 3685-3693, 1967
Printed in U.S.A.

Studies on the Chemistry of Hemoglobin

II. THE EFFECT OF SALTS ON THE DISSOCIATION OF HEMOGLOBIN INTO SUBUNITS*

(Received for publication, November 22, 1966)

GUIDO GUIDOTTI

From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

SUMMARY

The ability of several salt solutions to dissociate the tetrameric form of the hemoglobin molecule into dimers has been studied by measuring the osmotic pressure of solutions of hemoglobin. Under most circumstances, the dissociation reactions had the normal dependence on concentration of hemoglobin, and thus values of the dissociation constants could be estimated. In all the solvents studied here, the sequence of decreasing stability of the tetramer was deoxyhemoglobin > CO hemoglobin > oxyhemoglobin ≥ cyanmethemoglobin. This result was interpreted as providing further evidence for differences in conformation, not only between the liganded hemoglobins and deoxyhemoglobin, but also among the various liganded hemoglobins. Similar studies done with iodoacetamide-treated and *N*-ethylmaleimide-treated hemoglobins indicated that the latter derivative differs in conformation from normal hemoglobin whereas the former does not, by these criteria. Finally, the ability of the various salts to promote dissociation did not depend only on their contribution to the ionic strength of the solvent but also, and in some cases in large part, depended on specific interactions between the salt ions and the protein molecules. The order of effectiveness of the salts in dissociating the hemoglobin tetramers was NaI > guanidiniuni chloride > $NaClO_4$ > Cl_3 > NaCl ≅ CH_3COONa.

tetrameric molecule of human hemoglobin, $\alpha_2\beta_2$, is in equilibrium with its subunits according to the equation:

$$\alpha_2\beta_2 \underset{K_1}{\rightleftharpoons} 2\alpha\beta \underset{K_2}{\rightleftharpoons} 2\alpha + 2\beta \qquad (1)$$

all solvent conditions, for a review of the literature, see [illegible], Konigsberg, and Craig (1) and Riggs (2). The [illegible] of the constants, K_1 and K_2, depend on the pH, ionic strength, salt composition, and dielectric constant of the solvent.

* This work was supported by United States Public Health Service Grant HE 08881 and by National Science Foundation Grant GB [illegible].

The effect of the ionic strength of the solvent on the first dissociation reaction in Equation 1 has been investigated in several laboratories over the past 20 years (3-13), and most of these studies have indicated that dissociation into dimers is favored by an increase in the ionic strength of the solvent.

In spite of the fairly ample evidence on the nature of this reaction, dissociation by salts, there remain two points which require further comment.

The first point is concerned with the actual reaction which describes the equilibrium

$$\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$$

and stems from the inconsistencies in the data obtained in several laboratories on the concentration dependence of the dissociation reaction. Thus, while Rossi Fanelli, Antonini, and Caputo (6), Benesch, Benesch, and Williamson (7), and Benesch, Benesch, and Macduff (8) find that the dissociation reaction, although reversible, does not obey an equation of the type 1 → 2 (the weight average molecular weights are not dependent on hemoglobin concentration), Benhamou *et al.* (5) and Kirshner and Tanford (10) find that the equilibrium, up to a protein concentration of 0.7 g/100 ml, obeys the normal equation for dependence on solute concentration.

The second point deals with the effect of the state of the iron atom on the values of the dissociation constants for the tetramer-dimer equilibria in salts. On the one hand, Benesch *et al.* (7, 8) and Briehl (11) found that deoxyhemoglobin[1] is more highly aggregated than oxyhemoglobin in 2 M NaCl, in contrast to Rossi Fanelli *et al.* (6), who find no difference in the degree of aggregation of these two types of hemoglobin. However, there does seem to be agreement on the point that dissociation at acid pH is dependent on oxygenation (10, 11, 14, 15). On the other hand, one may infer from the data of Kirshner and Tanford (10) who compared human carbonmonoxyhemoglobin with bovine ferrihemoglobin, that the various liganded hemoglobins are similar in the ability to dissociate into dimers. This inference is supported by the data of Wyman *et al.* (see Reference 16) on the

[1] Deoxyhemoglobin designates hemoglobin in which the heme groups are not combined with any ligand. Thus, deoxyhemoglobin exists as dimers (deoxydimers) and as tetramers (deoxytetramers).

dissociation of oxy- and carbonmonoxyhemoglobins at acidic pH values.

However, I have recently pointed out (17) that there is evidence to suggest that not only does deoxyhemoglobin differ in conformation from the liganded hemoglobins (a hypothesis that is now generally accepted (16, 18)), but that the various liganded hemoglobins also differ from one another, albeit slightly, in conformation. It therefore is of interest to determine whether or not all these conformational variations may be reflected in the ability of the various hemoglobins to dissociate into dimers.

In this report I present the data obtained by measuring the osmotic pressures of deoxyhemoglobin and of various liganded hemoglobins in several salt solutions, with a view to distinguishing the effects on the dissociation reaction attributable to the ionic strength of the solvent from those due to the specific ions of the salt. The data presented here indicate that indeed the various liganded hemoglobins differ in conformation from one another and from deoxyhemoglobin, and that the tetramer-dimer equilibria obey the normal equation for dependence on solute concentration.

EXPERIMENTAL PROCEDURE

Materials—Solutions of normal adult human hemoglobin, of *N*-ethylmaleimide-treated and iodoacetamide-treated human hemoglobins, and of human cyanmethemoglobin were prepared as described in Paper I of this series (19). Solutions of horse hemoglobin were prepared from fresh, defibrinated horse blood, obtained through the courtesy of the Commonwealth of Massachusetts Department of Public Health Diagnostic Laboratories, by the methods previously described (20). EM[2]-treated and iodoacetamide-treated horse hemoglobin were prepared by the procedure used for human hemoglobin (21). The contents of *S*-succinylcysteine and of *S*-carboxymethylcysteine were 0.83 to 0.87 and 0.94 to 0.98 residue per $\alpha\beta$ molecule, respectively. The stoichiometry of the reaction (1 residue of cysteine per $\alpha\beta$ unit reacted) was judged to be sufficient proof of the specificity of these alkylation reactions with horse hemoglobin, and no further identification was attempted.

All the hemoglobin solutions were stored at a solute concentration of 10 g/100 ml at 2°, and they were used within 6 days of preparation.

Oxygen, carbon monoxide, and nitrogen were obtained from The Matheson Company, Inc., East Rutherford, New Jersey. The latter gas, prepurified by the company, was further purified by passage through a train of four gas washing bottles containing vanadous sulfate (two bottles), 1 N NaOH, and water (8, 22).

The inorganic salts were analytical grade reagents, used without further purification. Guanidine hydrochloride was prepared from guanidine carbonate by the method of Anson (23).

Methods—The concentration of hemoglobin was estimated spectrophotometrically after conversion to cyanmethemoglobin. The molar extinction coefficient of this derivative, based on a molecular weight of 64,450, is 4.6×10^4 M^{-1} cm^{-1} at 540 mμ (24).

The osmotic pressures were measured in a high speed membrane osmometer (Mechrolab division of Hewlett Packard, model 503), equipped with temperature control, at 20°. In this instrument equilibrium across the membrane was reached within 10 min after the sample was introduced. Attainment of equilibrium was determined by monitoring the rate of change of osmotic pressure on a recorder. The membranes were obtained from the Carl Schleicher and Schuell Company (type B-19 membrane filters, 42 mm in diameter). The membranes, which were stored in 20% ethanol at 2°, were washed with water and then soaked in the appropriate solvent for 6 hours before use.

For solutions containing CO hemoglobin,[2] the solvent was saturated with CO before it was used to fill the solvent side of the chamber that held the membrane, and the solution side of the membrane chamber was kept filled with CO-saturated solvent between measurements.

For solutions containing deoxyhemoglobin, the solvent was saturated with N_2 before it was placed on the solvent side of the membrane chamber. Then large volumes of solvent saturated with N_2 were flushed across the solution side of the membrane while a stream of N_2 was blown gently over the inlet tube for the solution. Before a series of measurements was started, a 10-ml sample of carboxypeptidase A-treated deoxyhemoglobin (25), which has a high affinity for O_2, was flushed through the compartment for solution in order to scavenge any O_2 in the solvent. Samples of deoxyhemoglobin were then transferred anaerobically with syringes into the solution compartment. During the entire course of measurements with deoxyhemoglobin, the N_2 barrier was kept in operation over the inlet tube for the solutions.

Measurements were made on solutions of hemoglobin prepared by diluting the concentrated stock solutions of hemoglobin in the appropriate solvent with the same solvent. The compositions of the solvents are stated in the tables and figures. After each osmotic pressure determination the solutions were removed from the instrument and the pH, protein concentration, and visible spectrum were determined.

Two types of controls were used to check for the reversibility of the dissociation reactions studied here. The first was designed to show that the effect of strong salt solutions on liganded hemoglobin is reversible. The concentrated stock solutions of hemoglobin in the appropriate solvents (the solvents examined were 0.4 M and 1 M $MgCl_2$ and 1 M and 2 M NaCl) were diluted (at least 20 times) with solutions of NaCl so that the final ionic strength was 0.2. The osmotic pressures of these solutions of hemoglobin were determined, and were found to correspond to those of solutions of hemoglobin which had not been exposed to solvents of high ionic strength. The second type of control was done to check for the reversibility of the molecular weight changes attending oxygenation and deoxygenation. Here solutions of deoxyhemoglobin in the appropriate solvent were allowed to oxygenate, and the osmotic pressures of these solutions were found to be identical with those of solutions of oxyhemoglobin which had never been deoxygenated (see Fig. 9 of Paper III of this series (26)).

The values for osmotic pressure, measured in centimeters of solvent, were reproducible to ±0.02 cm. Therefore the variation in the values of π/RTc is at worst ±5% at the lowest concentration of solute (0.1 g/100 ml) and ±0.5% at the highest concentrations of solute (≥1 g/100 ml). Three or four determinations of the osmotic pressure were made at each concentration of solute with different samples of solution.

RESULTS

The measurements of the osmotic pressure of the hemoglobin solutions can be represented by the equation

[2] The abbreviations used are: EM, *N*-ethylmaleimide; CO hemoglobin, carbonmonoxyhemoglobin.

$$\frac{\pi}{RTc} = \frac{1}{\bar{M}_n} + Bc \qquad (2)$$

where π is the pressure, in centimeters of solvent, c is the concentration of solute, in grams per 100 ml, B is an empirical parameter, R is the gas constant, $\bar{M}_n$ is the number average molecular weight, and T is the absolute temperature.

For a nonideal nonassociating system the significance of the parameter B has been developed (see Edsall (27)), and its value can be found experimentally from the limiting slope of the plot of π/RTc with respect to c.

For a nonideal associating system of the type $A_2 \rightleftharpoons 2A$, where $K = (c_A)^2/(c_{A_2})$, Equation 2 can be written as

$$\frac{\pi}{RTc} = \frac{1}{M_{A_2}}\left[1 + \frac{K}{2c}\left(-1 + \sqrt{1 + \frac{4c}{K}}\right)\right] + B'c \qquad (3)$$

if the molecular weight of the dimer, M_{A_2}, is known. Under the conditions used here,[2] the parameter B' will represent mainly the excluded volume terms for both the dimer and the monomer, and hence will vary as the weight fraction of these species varies, as a function of the total solute concentration (28). If it is assumed that the value of B' does not vary very much as a function of c, then the values of the dissociation constants, K, can be obtained by fitting the experimental curves (π/RTc with respect to c) with assumed values of K and B'. In practice, the experimental curves were compared with a series of theoretical curves constructed for a given value of the constant K and a succession of values for the parameter B'. The probable precision of the values of the dissociation constant obtained by this method is $\pm 10\%$. In a few instances, the graphical method described by Adams (29) was used to evaluate the dissociation constants.

Fig. 1 shows the osmotic pressure data for solutions of CO-, oxy-, and deoxyhemoglobins at low ionic strength (in a solvent 0.2 M in NaCl, pH 7). The number average molecular weights reported in Table I were obtained by extrapolation of the lines to zero protein concentration. Since the hemoglobin tetramer is in equilibrium with its subunits even under these solvent conditions, these values for the molecular weights clearly represent those characteristic of the system at low, but not at zero, protein concentration. It is not surprising that the values obtained are lower than that calculated from the amino acid composition (64,500), because there is a small but appreciable amount of dimer present at these concentrations of solute (approximately 10 to 15% by weight, of the hemoglobin is in the form of dimers). Fig. 2 shows the results of measurements done at very low concentrations of CO hemoglobin in this solvent. The tendency for the curve to curve upward is apparent. The value of the dissociation constant estimated with the use of the value of B' given in Table I is $K = 5 \times 10^{-6}$ M. This is closely comparable to the value 4×10^{-6} M obtained by Ackers and Thompson (30) and the value (2.5×10^{-6} M) estimated from the data of Kershner and Schroeder (10). For a solution of CO hemoglobin which is

[2] The solvents used in these studies had pH values close to the isoelectric point of human hemoglobin (pH 6.8 to 7.0) and ionic strength values greater than 0.2 (I = 0.2 to 0.5). Therefore, the term $-2B'c$ can be assumed to make a negligible contribution to the value of B'. The term which depends on the protein-salt, the salt-salt interactions must necessarily have an appreciable value in view of the specific ion effects described in this paper. However, for the present purposes it is not considered here; it will be the subject of a subsequent communication.

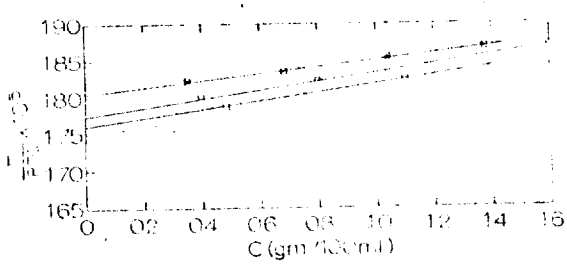


FIG. 1. Osmotic pressure data for hemoglobins in 0.2 M NaCl, pH 7. ⊠, oxyhemoglobin; ⊙, CO hemoglobin; □, deoxyhemoglobin.

TABLE I

Molecular weights of hemoglobin in 0.2 M NaCl, at pH 7 and 20°

Type of hemoglobin	Molecular weight	K
	g/mole	*mole/ml*
Oxyhemoglobin	55,100	4.7×10^{-6}
CO hemoglobin	56,100	6.1×10^{-6}
Deoxyhemoglobin	57,000	5.9×10^{-6}

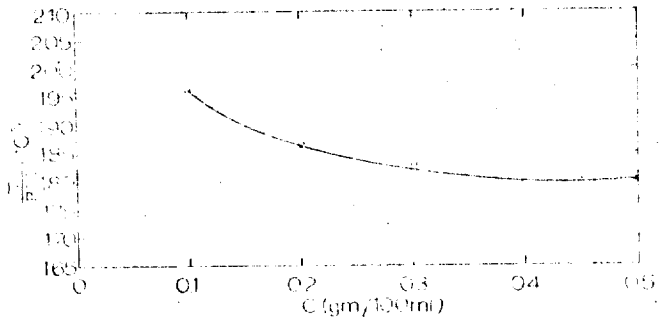


FIG. 2. Osmotic pressure data for CO hemoglobin in 0.2 M NaCl, pH 7.

0.4 g/100 ml, the degree of dissociation is 0.13 when the given value of the dissociation constant is used. This value, which accounts for the values of the molecular weights shown in Table I (see also the value in parentheses (above)), is closely comparable to the one estimated by Kawahara, Kershner, and Tanford (12) for CO hemoglobin (0.4 g/100 ml) in water or dilute salt solutions (the degree of dissociation is 0.06 and $K = 1 \times 10^{-6}$ M).

The differences between the values obtained for the three types of hemoglobin shown in Table I, which become more evident in the next series of experiments, suggest that the values of the dissociation constants for all three types of hemoglobin are different from one another, and that they decrease in the sequence, oxyhemoglobin > CO hemoglobin > deoxyhemoglobin.

Effect of Ionic Strength. Fig. 3 shows the osmotic pressure data obtained with solutions of oxyhemoglobin containing various concentrations of NaCl and MgCl$_2$. The *points* represent the experimental data, and the *lines* are the theoretical curves calculated with the values of the dissociation constants and of the interaction parameters shown in Table II for the solvent conditions for which the values of B' and K are given.

The *dashed line* shows the behavior which would be expected if the dissociation proceeded directly from tetramers to monomers

for the solution of oxyhemoglobin in 0.4 M $MgCl_2$. Since the experimental *points* fall very nicely on the theoretical curve for the dissociation of tetramers to dimers and do not deviate toward the *dashed line*, I conclude that under the solvent conditions in which it is possible to evaluate the values of K and B there is no appreciable dissociation to monomers.

The agreement between the calculated and the experimental curves up to a protein concentration of 1.2 g/100 ml indicates that the dissociation reaction (tetramers to dimers) obeys the normal equation for dependence on protein concentration. The values for the dissociation constants (Table II) are comparable to those obtained by Kirshner and Tanford (10), who measured the sedimentation coefficients of hemoglobin solutions in these same salts.

The data clearly indicate the striking difference in the ability of these two salts to promote dissociation ($MgCl_2$ is approximately 30 times more effective than NaCl at the same ionic strength). This effect must therefore be related specifically to the Mg^{++} ion.

Effect of Different Salts—Fig. 4 shows the osmotic pressure

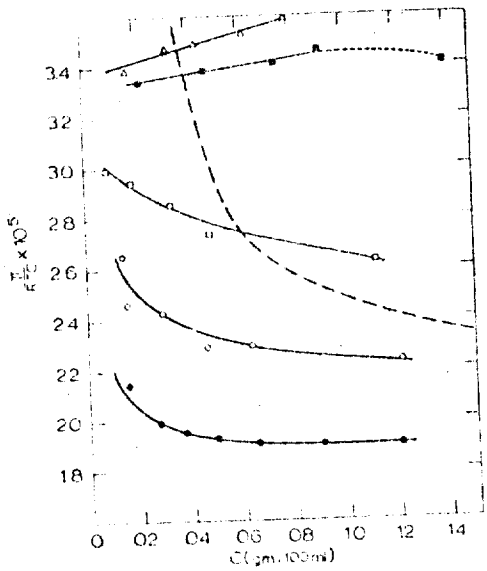


FIG. 3. Osmotic pressure data for solutions of oxyhemoglobin in NaCl and $MgCl_2$, pH 7. △, 1 M $MgCl_2$; ▽, 1 M $MgCl_2$, horse hemoglobin; ◐, 0.4 M $MgCl_2$; ○, 2 M NaCl; and ●, 1 M NaCl. All experiments were done with human hemoglobin, except for the one noted. The *dashed line* is a theoretical curve calculated for a tetramer to monomer dissociation reaction.

TABLE II

Tetramer to dimer dissociation constants for oxyhemoglobin in solvents at pH 7 and 20

Solvent	K	B
	M × 10^{5}	mole ml/g²
0.4 M $MgCl_2$	60	12.5 × 10^{-5}
1 M $MgCl_2$	> 1000	
1 M NaCl	2	16.8 × 10^{-5}
2 M NaCl	12	17.4 × 10^{-5}
1 M CH_3COONa	2	16.8 × 10^{-5}
1 M $NaClO_4$	> 1000	
1 M NaI	> 1000	

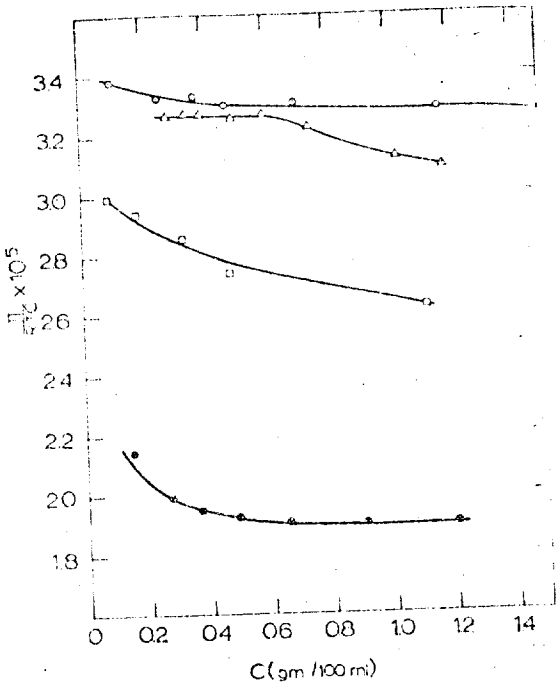


FIG. 4. Osmotic pressure data for oxyhemoglobin in solvents with $\Gamma/2 \geq 1$ and pH 7. ○, 1 M NaI; △, 1 M $NaClO_4$; ⊟, 0.4 M $MgCl_2$; ◐, 1 M NaCl and 1 M CH_3COONa.

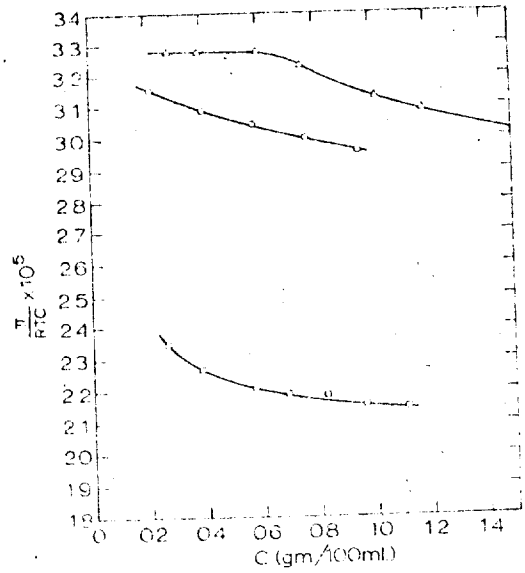


FIG. 5. Osmotic pressure data for hemoglobins in 1 M $NaClO_4$, pH 7. △, oxyhemoglobin; ○, CO hemoglobin; and ⊟, deoxyhemoglobin.

data obtained with oxyhemoglobin in a variety of salt solutions, all at an ionic strength of 1 and at pH 7. The curves for the solutions, 1 M NaI and 1 M $NaClO_4$, and the curves in Fig. 5 for the solution, 1 M $MgCl_2$, indicate that dissociation has proceeded beyond the stage of $\alpha\beta$ dimers to that of the monomers in these solutions of *liganded hemoglobin*. I have not attempted here to sort out the two successive dissociation processes, so the values of the dissociation constants shown

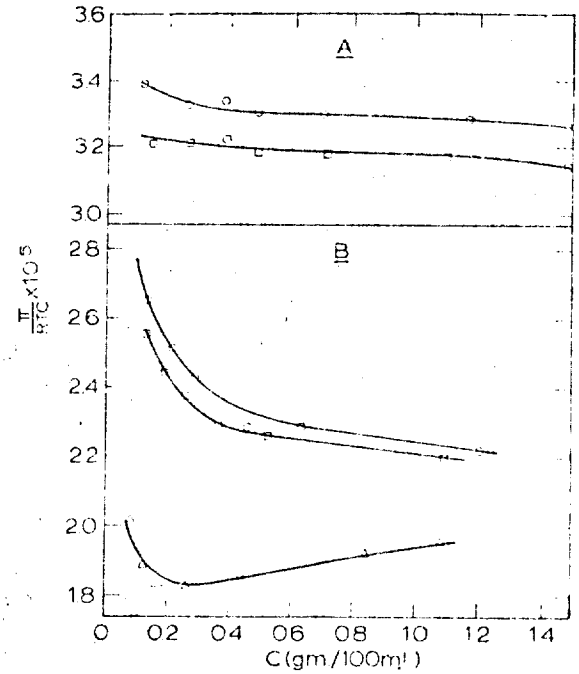


FIG. 6. Osmotic pressure data for hemoglobins in 1 M NaI (A) and in 2 M NaCl (B), pH 7. ○, oxyhemoglobin; □, CO hemoglobin; and △, deoxyhemoglobin.

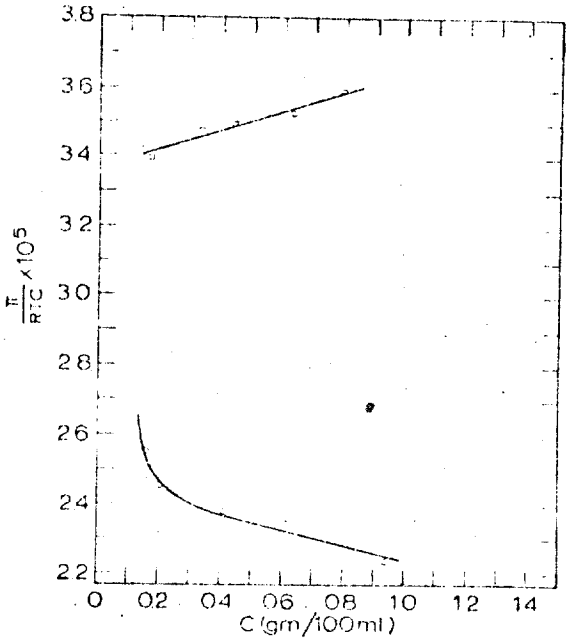


FIG. 8. Osmotic pressure data for oxyhemoglobin and cyanmethemoglobin (□) and for deoxyhemoglobin (○) in 1 M $MgCl_2$, pH 7.

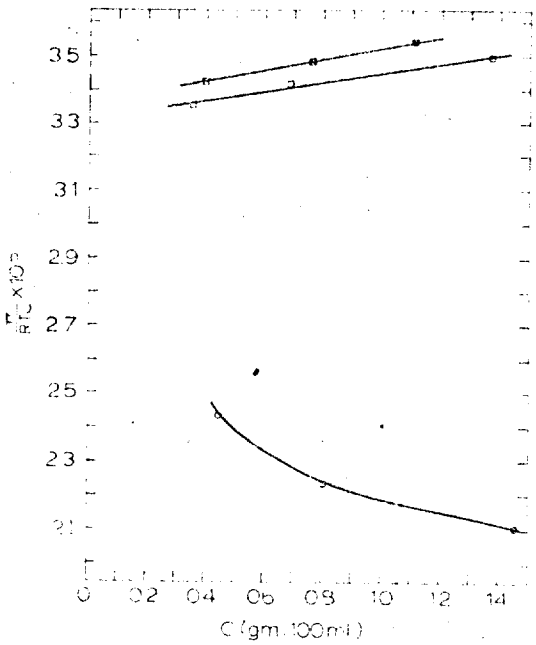


FIG. 7. Osmotic pressure data for hemoglobins in 1.74 M guanidinium chloride, pH 7. ●, oxyhemoglobin; □, CO hemoglobin, ○, deoxyhemoglobin.

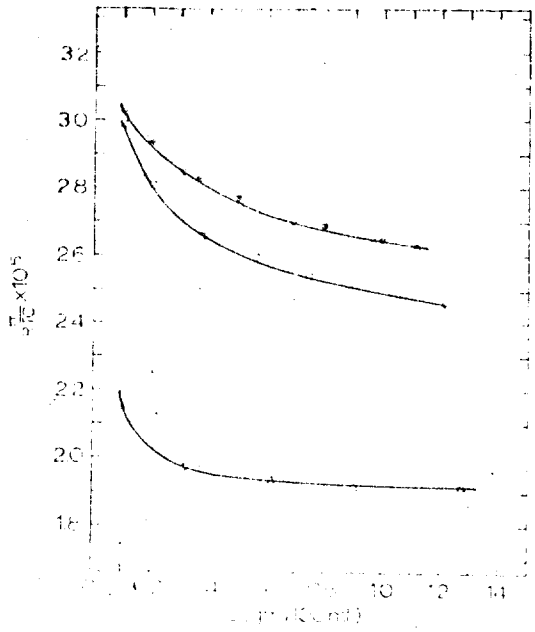


FIG. 9. Osmotic pressure data for hemoglobins in 0.1 M $MgCl_2$, pH 7. ●, cyanmethemoglobin; □, oxyhemoglobin; □, CO hemoglobin; △, deoxyhemoglobin.

[illegible] conditions are only very approximate and indicate the [illegible] magnitude.[illegible] Even with this restriction, the data [illegible] indicate that the order of effectiveness as disso-

[illegible] effect of these and other similar salts on the dissociation of hemoglobin into dimers and monomers is at present under study here.

ciating agents is NaI > $NaClO_4$ > $MgCl_2$ > NaCl ≥ CH_3COONa at an ionic strength of 1. Since it is the Mg^{++} ion that is effective in promoting dissociation, it seems reasonable to compare the salts at the same molar concentration of salt. The order is NaI > $MgCl_2$ > $NaClO_4$ > NaCl > CH_3COONa (Figs. 3 and 4). The finding that NaCl and CH_3COONa have the same effect on the integrity of the hemo-

globin tetramer at neutral pH is not surprising in spite of the report by Chiancone and Gilbert (31) that acetic acid favors the dissociation of the tetramer. At neutral pH the concentration of acetic acid in a solution 1 M in sodium acetate is only 5×10^{-8} M, and thus can be considered to be negligible.

Effect of Ligand.—Figs. 5 to 9 show the osmotic pressure data obtained in several different salt solutions with cyanmethemoglobin, CO hemoglobin, oxyhemoglobin, and deoxyhemoglobin. The values for the dissociation constants estimated from these data are shown in Table III. In all cases, the stability of the deoxyhemoglobin tetramer is much greater than that of the liganded hemoglobins. In fact, the dissociation of deoxyhemoglobin tetramers does not proceed beyond the stage of dimers in any of the solvents used here, as judged by the agreement between the theoretical *curves* and the experimental *points*. In

TABLE III

Tetramer to dimer dissociation constants for deoxyhemoglobin and liganded hemoglobins in solvents at pH 7 and 20°

Type of hemoglobin	K				
	$NaClO_4$, 1 M	Guanidine-HCl, 1.71 M	$MgCl_2$, 1 M	$MgCl_2$, 0.4 M	NaCl, 2 M
	M × 10				
Deoxyhemoglobin	6	15	9	2	0.5
CO hemoglobin	L[a]	L		30	7.5
Oxyhemoglobin	L	L	L	60	12
Cyanmethemoglobin			L	60	

[a] L indicates values too large to measure with these data. In all cases, however, the values of the dissociation constants for CO hemoglobin are smaller than those for oxyhemoglobin.

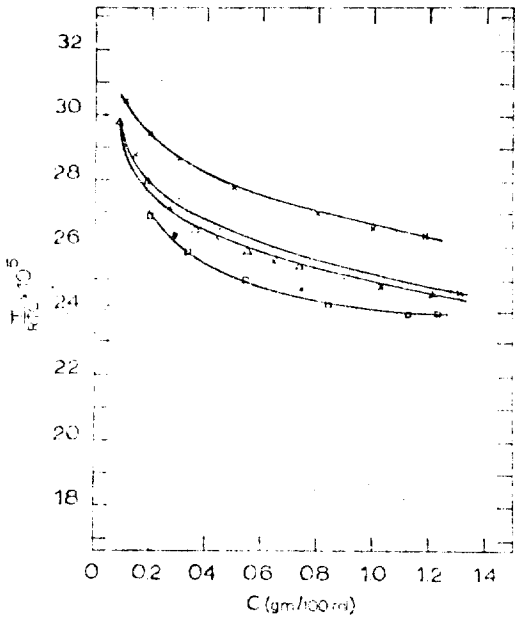


FIG. 10. Osmotic pressure data for modified hemoglobins in 0.4 M $MgCl_2$, pH 7. (*upper curve*), cyanmethemoglobin and iodoacetamide-treated cyanmethemoglobin; •, EM-treated cyanmethemoglobin; ×, CO hemoglobin, ⊗ (*lower curve*), iodoacetamide-treated CO hemoglobin, ⊡, EM-treated CO hemoglobin.

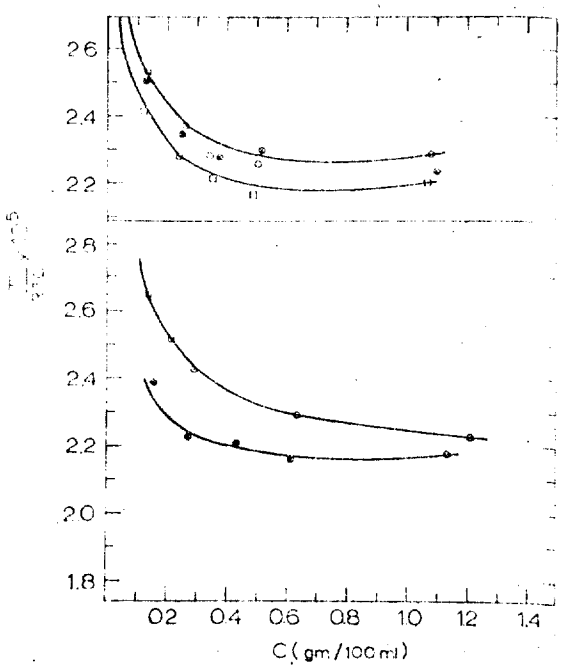


FIG. 11. Osmotic pressure data for modified hemoglobins in 2 M NaCl, pH 7. *Upper:* ⊙, CO hemoglobin; ⊗, iodoacetamide-treated CO hemoglobin; and ⊡, EM treated CO hemoglobin. *Lower:* ⊙, oxyhemoglobin; ⊗, EM-treated oxyhemoglobin.

TABLE IV

Dissociation constants for treated hemoglobins in 0.4 M $MgCl_2$ and 2 M NaCl, pH 7 and 20°

Type of hemoglobin	K	
	0.4 M $MgCl_2$	2 M NaCl
	M × 10⁵	
Oxyhemoglobin	60	12
EM-treated oxyhemoglobin		5
CO hemoglobin	30	7.5
Iodoacetamide-treated CO hemoglobin	30	7.5
EM-treated CO hemoglobin	25	5
Cyanmethemoglobin	60	
Iodoacetamide-treated cyanmethemoglobin	60	
EM-treated cyanmethemoglobin	35	

some of the solvents, the liganded hemoglobins do behave as though the dissociation reaction must include the presence of an appreciable concentration of monomers. In these circumstances, I have not tried to evaluate values of the various dissociation constants. However, there are also present small but consistent differences between the various liganded hemoglobins, whose stability decreases in the order, COHb > oxyhemoglobin ≧ cyanmethemoglobin.

Effect of Chemical Modification.—Figs. 10 and 11 show the data obtained with normal, iodoacetamide-treated and EM-treated hemoglobins, in 0.4 M $MgCl_2$ and 2 M NaCl. In both salt solutions there is no appreciable difference between the iodoacetamide treated and the normal hemoglobin, but the EM-treated hemoglobin appears to be more stable than the latter two types of hemoglobin (Table IV).

DISCUSSION

On the basis of the data presented in previous communications (1, 13, 21, 32) and in Paper III of this series (26), the reactions which describe the dissociation of human hemoglobin are those shown in Equation 1, which was proposed by Vinograd and Hutchinson (33).

Here the main interest is in the dissociation of the tetrameric molecule into dimers

$$\alpha_2\beta_2 \underset{K}{\rightleftharpoons} 2\,\alpha\beta$$

In all the salt solutions reported here, the dissociation reaction for deoxyhemoglobin could be described by the expression $K = \alpha^2 c/(1 - \alpha)$, where K is the dissociation constant, in grams per 100 ml; c is the concentration of hemoglobin, in grams per 100 ml, and α is the weight fraction of hemoglobin in the state of the dimer. The same is true for the liganded hemoglobins in the two solvents (2 M NaCl and 0.4 M $MgCl_2$) in which dissociation took place only to the stage of dimers. This agreement between the experimental data and the normal equation for dependence on protein concentration held up to a concentration of 1.2 g/100 ml. I am therefore unable to explain the results of Benesch *et al.* (7, 8), and of Rossi-Fanelli *et al.* (6) on the lack of concentration dependence.

Effect of Ionic Strength and of Specific Salt Ions. The data presented here should be considered together with the results of Kawahara *et al.* (12) and Kirshner and Tanford (10) on the dissociation of hemoglobin by urea and salts, and in conjunction with the extensive data of Robinson and Jencks (34, 35) on the effect of urea and salt solutions on the activity coefficient of acetylhetraglycine ethyl ester and with those of Nagy and Jencks (36) on the depolymerization of F-actin by salt solutions.

The order of effectiveness of the salts in the dissociation of the hemoglobin tetramers is NaI > guanidinium chloride ≥ $NaClO_4$ > $MgCl_2$ > NaCl ≅ CH_3COONa (Table II). If the groups involved in the area of contact between $\alpha\beta$ subunits were simply ionic groups, one would expect that the activity coefficients of the groups that are exposed to the solvent upon dissociation would depend mainly on the value of the ionic strength, and thus would be independent of the type of salt. Instead, the ability of salts to dissociate the hemoglobin tetramer follows the Hofmeister series of ions, and is similar to that for the depolymerization of F-actin (36) and for decreasing the activity coefficient of acetylhetraglycine ethyl ester (35). I therefore conclude, in agreement with Kawahara *et al.* (12), that the surfaces of the dimers which come in contact contain groups that are stabilized by salt ions and urea, which, on the basis of the data of Robinson and Jencks (35), are most likely peptide and amide groups. It is assumed that the effect of the solutions of NaCl is due mainly to the interaction of this salt with charged groups, so that this therefore is in the effect of ionic strength. The difference in ability to dissociate hemoglobin between the various salts at the same ionic strength is a measure of the interaction between the specific ions and the peptide and amide groups in the contact surface.

[illegible] the free energy changes which result from the [illegible] of a single site on acetylhetraglycine ethyl ester [illegible] solutions (35), it can be estimated that in oxyhemoglobin [illegible] groups of the type acetylhetraglycine ethyl ester, are removed from contact with the solvent when the dimers associate. This value is to be compared with that estimated by Kawahara *et al.* (12), whose data indicate the involvement of 10 to 20 amide groups in this interaction.

TABLE V

Standard free energy changes for tetramer to dimer dissociation of hemoglobin in 2 M NaCl and 0.4 M $MgCl_2$, pH 7 and 20°

Type of hemoglobin	ΔG°	
	2 M NaCl	0.4 M $MgCl_2$
	kcal/mole	
Deoxyhemoglobin	7.1	6.3
CO hemoglobin	5.5	4.7
Oxyhemoglobin	5.2	4.3
Cyanmethemoglobin		4.3

Effect of Ligand. A striking feature of these results is the consistent difference in the stability of the hemoglobin tetramers combined with different ligands in all the salt solutions. The order of decreasing stability (Table III) is deoxyhemoglobin > CO hemoglobin > oxyhemoglobin ≥ cyanmethemoglobin. The values for the dissociation constants provide a measure of the free energy of interaction between the subunits, which is shown in Table V.

The data for deoxyhemoglobin as compared to oxyhemoglobin (the former being more stable, by 2000 cal per mole, than the latter) agree with the general trend of those obtained by Benesch *et al.* (7, 8) and Bricht (11), but are not in agreement with those of Rossi-Fanelli *et al.* (6), who found no difference between these two types of hemoglobin in their ability to dissociate in salt solutions. I can provide no explanation for this discrepancy.

There is another apparent inconsistency between the results presented here and the data of Kirshner and Tanford (10), who found a similar dissociation behavior for human CO hemoglobin and bovine ferrihemoglobin. However, CO hemoglobin and ferrihemoglobin (which was not studied here) may in fact be identical in their ability to dissociate into dimers. Furthermore, the conclusions of these authors on the identical behavior of liganded hemoglobins rest on the hypothesis that human and bovine hemoglobins combined with the *same* ligand are identical in their stability in salt solutions. There is no reason to believe that this hypothesis is true.

The simplest explanation for the differences described here in the free energy changes for the dimerization process is that the conformations of the various hemoglobin *dimers* combined with different ligands are different, one from the other.

I emphasize here that the conformational differences between the various forms of hemoglobin are small structural changes situated in the $\alpha\beta$ dimers. These must necessarily be brought about by the presence or the absence of a *specific* ligand at the sixth coordination position of the iron atom of the heme group on both chains of a dimer. Heard *et al.* (37) have recently proposed that the position of the iron atom relative to the plane of the heme may differ depending on the electronic state of the iron atom. Thus, as the bond length between the iron atom and the heme-linked histidine changes, [illegible] will be measured by different bonds and [illegible] represent variations in the bond length. [illegible] change, the structure of the dimer [illegible] to the heme linked histidine [illegible]

from its state in deoxyhemoglobin, and it would be altered differently by the different ligands. This results in a different conformation for the $\alpha\beta$ dimer for each type of dimer (deoxydimer ≠ oxydimer ≠ CO dimer, etc.). This conclusion can be related to the results of the experiments reported here, in Paper I of this series (19), and to the data in the literature in the following manner. It was shown previously (17, 19) that oxyhemoglobin, CO hemoglobin, and deoxyhemoglobin all differed one from the other in the velocity of reaction of the —SH group at Position 93 on the β chain (next to the heme linked histidine residue), but it was assumed that in the *dimers* of these various hemoglobins the reaction velocity was only slightly different. This result is taken as an indication of the *small* structural changes in the dimers of these hemoglobins. Now when the liganded dimers associate into tetramers, they do so without undergoing any further conformational change. Hence the reactivity of the reactive —SH group is the same for the dimers and the tetramers of these hemoglobins, and the free energy changes for association of the dimers are all closely grouped (within 400 cal per mole) although significantly different (and in the same order as the velocities of reaction of the —SH groups of these hemoglobins).

However, the structure of the *dimers* of deoxyhemoglobin is such that the contact surface between dimers is dramatically different from that between the dimers of the liganded hemoglobins, and is such that when the dimers associate they undergo a further conformational change. In this way I explain (*a*) the difference between the reactivity of the —SH group of the dimer of deoxyhemoglobin and that of the —SH groups of the tetramer (19), (*b*) the much greater stability of the tetramer of deoxyhemoglobin than of the tetramer of the liganded hemoglobins ("Results," above), (*c*) the different three dimensional structure of the crystal of deoxyhemoglobin as compared to those of the liganded hemoglobins (38, 39), and (*d*) the ample evidence for a dramatic difference between the conformation of the tetramer of deoxyhemoglobin and those of the tetramers of the liganded hemoglobins (16, 18).

The conclusion that the fundamental differences between the structure of deoxyhemoglobin and those of the liganded hemoglobins reside in the dimer is of course supported by the immunological work of Reichlin *et al.* (40, 41), which was done under conditions in which all the hemoglobin would be in the form of the dimers.[2] The conclusion that the structures of the various liganded $\alpha\beta$ dimers differ one from the other is not generally accepted. In fact, it has commonly been assumed that the liganded hemoglobins have identical conformations (see Reference 17).

It remains to be seen whether or not the small structural differences between the $\alpha\beta$ dimers of all the hemoglobins considered here (both deoxyhemoglobin and the liganded hemoglobins), which I consider to be necessarily present and which have not been seen so far by x-ray diffraction studies, will be found subsequently by diffraction work at higher resolution.

[2] The dissociation constant for deoxyhemoglobin in solutions of low ionic strength can be estimated to have an approximate value of 10^{-6} M, if the same difference between the dissociation constants of deoxyhemoglobin and the liganded hemoglobins that is present at elevated ionic strength (Table III) obtains at low ionic strength. With this value for the dissociation constant, at the concentrations of hemoglobin (0.05 to 0.5 μg per ml) used for the immunological work (40), the fraction of hemoglobin in the form of dimers varies from 0.97 to 0.8 N.

Effect of Chemical Modification.—There are small differences in the dissociation constants of EM-treated hemoglobin as compared with iodoacetamide-treated hemoglobin, which in this respect resembles normal hemoglobin. These differences can be interpreted, in accord with the discussion given above, as evidence for a difference in conformation between these two modified hemoglobins (the difference in the free energy of dissociation is about 300 cal per mole). It may be purely coincidental but is nevertheless in agreement with the reported observations on EM-treated hemoglobin, which shows marked variations in oxygenation parameters from normal hemoglobin (42, 43), one whose ability to dissociate into dimers differs from that of normal hemoglobin, and thus by inference has a conformation which differs from that of normal hemoglobin. On the other hand, iodoacetamide-treated hemoglobin, which resembles normal hemoglobin in conformation, is also nearly identical with the latter in its oxygenation properties (except for an increased oxygen affinity) (43).

Acknowledgment.—My best thanks are due to Professor John T. Edsall for his interest and his very helpful advice.

REFERENCES

1. Guidotti, G., Konigsberg, W., and Craig, L. C., *Proc. Nat. Acad. Sci. U. S. A.*, 50, 774 (1963).
2. Riggs, A., *Physiol. Rev.*, 45, 619 (1965).
3. Svedberg, T., and Pedersen, K. O., *The ultracentrifuge*, Clarendon Press, New York, 1940, p. 356.
4. Gerhardt, B., in F. J. W. Roughton and J. C. Kendrew (Editors), *Haemoglobin*, John Wiley and Sons, Inc., New York, 1949, p. 197.
5. Benhamou, N., Daune, M., Jacob, M., Luzzati, A., and Weill, G., *Biochim. Biophys. Acta*, 37, 1 (1960).
6. Rossi Fanelli, A., Antonini, E., and Caputo, A., *J. Biol. Chem.*, 236, 341 (1961).
7. Benesch, R. E., Benesch, R., and Williamson, M. E., *Proc. Nat. Acad. Sci. U. S. A.*, 48, 2071 (1962).
8. Benesch, R. E., Benesch, R., and Macduff, F., *Biochemistry*, 3, 1132 (1964).
9. Anslan, A., and Yphantis, D., *Abstracts of the Eighth Annual Meeting of the Biophysical Society, Chicago, Ill.*, WB 12.
10. Kirschner, A. G., and Tanford, C., *Biochemistry*, 3, 291 (1964).
11. Brewer, R. W., in T. W. Goodwin, J. T. Harris, and B. S. Hartley (Editors), *Structure and activity of enzymes*, Academic Press, New York, 1964, p. 171.
12. Kawahara, K., Kirschner, A. G., and Tanford, C., *Biochemistry*, 4, 1203 (1965).
13. Guidotti, G., and Craig, L. C., *Proc. Nat. Acad. Sci. U. S.*, 50, 46 (1963).
14. Rainey, H. M., Benesch, R. E., Benesch, R., and Jacobs, A. S., *Biochim. Biophys. Acta*, 74, 511 (1963).
15. Wyman, J., *Advances Protein Chem.*, 19, 223 (1964).
16. Rossi Fanelli, A., Antonini, E., and Caputo, A., *Advances Protein Chem.*, 19, 73 (1964).
17. Guidotti, G., *J. Biol. Chem.*, 240, 3924 (1965).
18. Benesch, R., and Benesch, R. E., *J. Mol. Biol.*, 6, 498 (1963).
19. Guidotti, G., *J. Biol. Chem.*, 242, 3673 (1967).
20. Hess, R. J., Konigsberg, W., Guidotti, G., and Craig, L. C., *J. Biol. Chem.*, 237, 1519 (1962).
21. Guidotti, G., and Konigsberg, W., *J. Biol. Chem.*, 239, 1174 (1964).
22. Meites, L., and Meites, T., *Anal. Chem.*, 20, 984 (1948).
23. Anson, M. L., *J. Gen. Physiol.*, 24, 399 (1941).
24. Drysdale, D. L., and Austin, J. H., *J. Biol. Chem.*, 142, 7 (1965).
25. Antonini, E., Wyman, J., Zito, R., Rossi Fanelli, A., and Caputo, A., *J. Biol. Chem.*, 236, PC63 (1961).
26. Guidotti, G., *J. Biol. Chem.*, 242, 3684 (1967).

2. Ebsell, J. T., in H. Neurath and K. Bailey (Editors), *The proteins, Vol. I, Part B*, 519, 1953.
3. Tanford, C., *Physical chemistry of macromolecules*, John Wiley and Sons, Inc., New York, 1961, p. 202; p. 290.
4. Adams, E. T., Jr., *Biochemistry*, **4**, 1655 (1965).
5. Ackers, G. K., and Thompson, T. E., *Proc. Nat. Acad. Sci. U. S. A.*, **53**, 342 (1965).
6. Chiancone, E., and Gilbert, G. A., *J. Biol. Chem.*, **240**, 3867 (1965).
7. Guidotti, G., in T. W. Goodwin, J. T. Harris, and B. S. Hartley (Editors), *Structure and activity of enzymes*, Academic Press, New York, 1964, p. 157.
8. Vinograd, J., and Hutchinson, W. D., *Nature*, **187**, 216 (1960).
9. Robinson, D. R., and Jencks, W. P., *J. Amer. Chem. Soc.*, **87**, 2462 (1965).
35. Robinson, D. R., and Jencks, W. P., *J. Amer. Chem. Soc.*, **87**, 2470 (1965).
36. Nagy, B., and Jencks, W. P., *J. Amer. Chem. Soc.*, **87**, 2480 (1965).
37. Hoard, J. L., Hamor, M. J., Hamor, T. A., and Caughey, W. S., *J. Amer. Chem. Soc.*, **87**, 2312 (1965).
38. Muirhead, H., and Perutz, M. F., *Nature*, **199**, 633 (1963).
39. Perutz, M. F., Bolton, W., Diamond, R., Muirhead, H., and Watson, H. C., *Nature*, **203**, 687 (1964).
40. Reichlin, M., Bucci, E., Antonini, E., Wyman, J., and Rossi-Fanelli, A., *J. Mol. Biol.*, **9**, 785 (1964).
41. Reichlin, M., Bucci, E., Wyman, J., Antonini, E., and Rossi Fanelli, A., *J. Mol. Biol.*, **11**, 775 (1965).
42. Riggs, A., *J. Biol. Chem.*, **236**, 1948 (1961).
43. Benesch, R., and Benesch, R. E., *J. Biol. Chem.*, **236**, 405 (1961).

APPLIED MICROBIOLOGY, Nov. 1969, p. 854-855
Copyright © 1969 American Society for Microbiology
Vol. 18, No. 5
Printed in U.S.A.

Effectiveness of Acetic Acid, Betadine, Amphyll, Polymyxin B, Colistin, and Gentamicin Against *Pseudomonas aeruginosa*

MARY HUDBERG AND JOHN K. MILLER

Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

Received for publication 31 July 1969

The in vitro effectiveness of three germicides and three chemotherapeutic drugs against hospital isolated strains of *Pseudomonas aeruginosa* was determined. Solutions of 1% acetic acid, 0.5% Amphyll, and 0.1% Betadine were bactericidal for six strains tested within 15 min at room temperature. Over a 3-year period at the Albany Medical Center Hospital, the percentages of strains of *P. aeruginosa* susceptible to polymyxin B, colistin, and gentamicin did not increase. During this 3-year period, polymyxin B and colistin were administered at the hospital but gentamicin was not.

Pseudomonas aeruginosa is an important cause of hospital-acquired infection, particularly in patients with diminished resistance (1). The mortality rate in *P. aeruginosa* bacteremia is greater than in other gram-negative rod bacteremias (3). Of the available chemotherapeutic drugs, only the polymyxins and gentamicin appear to be effective against *P. aeruginosa*.

Sources of infection include water environments (drains and taps), patients with *P. aeruginosa* infections, and fecal material from normal individuals or other patients (1). Respiratory *Pseudomonas* infections have resulted from contaminated intermittent positive pressure breathing apparatus (4), and urinary-tract infections have frequently resulted from catheterization (5).

Acetic acid has been used for decontamination of nebulization equipment (2) and for treating and preventing urinary bladder infection (6). In this study, the in vitro effectiveness of three disinfecting agents and of three chemotherapeutic drugs against hospital isolated strains of *P. aeruginosa* was examined.

MATERIALS AND METHODS

Germicidal agents. Acetic acid, Betadine solution (povidone iodine, the Purdue Frederick Co., Yonkers, N.Y.), and Amphyll (Lehn & Fink Products Corp., Bloomfield, N.J.) were tested for bactericidal activity against five environmental strains (from drains, a temperature gauge, and a breathing hose) and one blood culture isolate from the Albany Medical Center Hospital. The agents were diluted in sterile tap water. Dilutions of 18-hr brain cultures were prepared in sterile tap water. To the dilutions of germicidal agents, 0.1 ml of a culture dilution was added, to yield 10^5 to 10^6 colonies per ml. After 1-, 15-, and 60-min contact at room temperature, 0.05 ml of the mixture was plated on the surface of a blood-agar plate. The inoculum was spread over one-half the surface of the plate with the tip of the pipette used for measuring. The plates were incubated overnight at 36 C and the colonies were counted. For the purpose of this experiment, the agents were considered bactericidal at the concentration which allowed no growth on the plate. No inactivating agents were incorporated in the blood agar.

TABLE 1. *Bactericidal concentrations of acetic acid, Amphyll, and Betadine for P. aeruginosa*

Culture no.	Source	Bactericidal concn					
		Acetic acid		Amphyll		Betadine	
		15 min	1 hr	15 min	1 hr	15 min	1 hr
		%	%	%	%	%	%
008	Drain	1	1	0.5	0.5	0.1	0.05
009	Drain	>1	1	0.5	0.5	0.05	0.05
011	Drain	>1	1	0.5	0.5	0.05	0.05
012	Gauge	>1	1	0.5	0.5	0.1	0.05
030	Breathing hose	1	1	--[a]	-	-	
277	Blood culture	--	---	---	--	0.1	0.1

[a] Not tested.

Chemotherapeutic agents. Three groups of hospital isolates of *P. aeruginosa*, collected at the Albany Medical Center Hospital over a period of 3 years, were tested for their susceptibility to polymyxin B, colistin,

TABLE 2. *Colony counts obtained from mixtures of P. aeruginosa culture no. 012 with Betadine, Amphyll, and acetic acid*

Time after mixing	Betadine		Amphyll		Acetic Acid	
	0.05%	0.025%	0.25%	0.125%	1%	0.5%
1 min	>100	Confluent	75	ca. 200	Confluent	Confluent
15 min	10	>100	9	200	>100	>100
1 hr	0	7	7	50	0	11

TABLE 3. *Percentage of hospital strains of P. aeruginosa susceptible to polymyxin B, colistin, and gentamicin*

Date of isolation	No. of strains	Polymyxin B		Colistin		Gentamicin	
		50 units	300 units	2 μg	10 μg	2 μg	10 μg
		%	%	%	%	%	%
Aug.–Sept. 1964	101	86	99	2	96	26	99
Feb. 1967	100	88	100	0	100	48	99
Aug. 1967	188	100	100	8	100	49	98

and gentamicin. (Polymyxin B and colistin discs were from BBL; gentamicin discs were supplied through the courtesy of The Schering Corp., Bloomfield, N.J.).

RESULTS

The effectiveness of the disinfectants is summarized in Table 1. The concentration required for bactericidal action within 15 min was usually no greater than that required within 1 hr. A 1% solution of acetic acid was required, although Sanford (6) and Edmondson and colleagues (2) found 0.25% effective for decontamination and control of infection. Amphyll was effective at the 0.25% concentration recommended by the manufacturer. Betadine solution was bactericidal at 0.05%, although it is intended for use without dilution.

Since no inactivating agents were incorporated in the blood-agar, it was possible that small amounts of the bactericidal agents carried over when the mixtures were plated might have caused bacteriostasis. Table 2 shows that the effectiveness of solutions of Betadine and acetic acid varied with time; lower colony counts after 15 min and 1 hr suggest that carry-over is not an important factor after longer periods. Colony counts from Amphyll mixtures did not change significantly with time, indicating that carry-over of Amphyll in the inoculum may have been important.

Table 3 shows the percentage of strains susceptible to two levels of each chemotherapeutic drug among the three groups of isolates. There was no increase in the number of resistant strains during the 3-year period, in which 2,630 vials of polymyxin B (500,000 units/vial) and 350 vials of colistin (0.15 g/vial) were used. Gentamicin was not used at the hospital during this period.

LITERATURE CITED

1. Editorial. 1964. Hospital infection with *P. pyocyanea*. Brit. Med. J., 2:1019-1020.
2. Edmondson, L. B., J. A. Reinartz, A. K. Pierce, and J. P. Sanford. 1966. Nebulization equipment. A potential source of infection in Gram-negative pneumonias. Amer. J. Dis. Child. 111:357-360.
3. McHenry, M. C., W. J. Martin, and W. E. Wellman. 1962. Bacteremia due to Gram-negative bacilli. Review of 113 cases encountered in the five-year period 1955 through 1959. Ann. Intern. Med. 56:207-219.
4. McNamara, M. J., M. C. Hall, A. Balows, and E. B. Lieber. 1967. A study of the bacteriologic patterns of hospital infections. Ann. Intern. Med. 66:480-488.
5. Sanford, J. P. 1964. Hospital acquired urinary tract infections. Ann. Intern. Med. 60:903-914.
6. Sanford, J. P. 1966. Treatment and prevention of opportunistic infections of the urinary tract. Med. Times. 8:1162-1170.

Biosynthesis of Progesterone, Sterols, and Squalene from Acetate-1-^{14}C and Mevalonate-2-^{14}C by the Bovine Corpus Luteum *in Vitro**

J. Biol. Chem. 240(5): 1957-61, 1965

HERMIONE R. HELLIG AND KENNETH SAVARD†

From the Endocrine Laboratory, Departments of Medicine and Biochemistry, University of Miami School of Medicine, Miami 36, Florida

(Received for publication, November 19, 1964)

Although the role of acetate as a substrate for steroid biosynthesis in systems *in vitro* is now well established (1-4), the role of mevalonate is currently somewhat obscure. A number of conflicting reports on the utilization of the latter substance for steroidogenesis have appeared in the literature. The diversity of steroidogenic tissue preparations that have been tested for their ability to utilize mevalonate includes testicular homogenates (5), testis slices (6), adrenal minces and homogenates (7, 8), rat adrenal slices (9), adrenal adenoma (10), and ovarian minces (11). However, in some of these reports the omission of critical data, such as precise radioactivity measurements or criteria of radiochemical purity, makes it difficult to evaluate the consequently ambiguous results.

The utilization of mevalonate in the bovine corpus luteum has not previously been rigorously investigated (3). This compartment of the ovary has the experimental advantage of producing only two steroids, the principal one being progesterone, and 20β hydroxy-Δ^4-pregnen-3-one comprising the minor product.

The conversion of labeled cholesterol to progesterone in the corpus luteum has been amply shown through the use of slices (12), homogenates (13), and mitochondrial preparations (14, 15). The utilization of acetate-1-^{14}C as a substrate for squalene, sterol, and progesterone synthesis in this tissue has also been shown (3, 4, 16), and the extent of its utilization for steroidogenesis has been shown to be increased in the presence of the pituitary gonadotropin, luteinizing hormone (4).

In view of the obligatory role of mevalonate in the biosynthesis of cholesterol from acetate in liver (17), it was of interest to investigate more fully the utilization of mevalonate-2-^{14}C for progesterone biosynthesis in the bovine corpus luteum. The incorporation of mevalonate-2-^{14}C into other lipid constituents was also assessed. Radioactive leucine incorporation into progesterone was investigated because of the possibility of this amino acid acting as a precursor for mevalonate.

EXPERIMENTAL PROCEDURE

Materials

Chemicals—Solvents were of reagent grade and distilled before use. The petroleum ether used had a boiling range of 40-50° and was purified by successive shakings with concentrated H_2SO_4, dilute $KMnO_4$, 10% Na_2CO_3, and distilled water. It was then dried over $CaCl_2$, fractionally distilled, and stored over metallic sodium. Alumina (aluminum oxide, Merck and Company), was made into a slurry successively with methyl formate and with anhydrous methanol, dried at 105° overnight, and stored in a desiccator.

Progesterone, m.p. 126.5-127.5°, was a gift from Syntex, S.A., Mexico City. Squalene (98% pure), cholesterol, m.p. 148-150°, and a 2:1 mixture of lanosterol and dihydrolanosterol were purchased from commercial sources.

Gonadotropin—Luteinizing hormone (ovine, NIH-LH-S1) was generously provided by the Endocrine Study Section of the National Institutes of Health.

Radioactive Compounds—The tritiated compounds used for measurement of recoveries, progesterone-7-^{3}H (specific activity, 952 μc per μmole) and cholesterol-7-^{3}H (specific activity, 296 μc per μmole), were purchased from New England Nuclear Corporation. Acetate-1-^{14}C (specific activity, 20 μc per μmole) was obtained from Nuclear Research Chemicals, Inc., and DL-leucine-2-^{14}C (specific activity, 1.11 μc per μmole) was obtained from Nichem, Inc., Bethesda, Maryland.

Two different preparations of DL-mevalonate-2-^{14}C were used. DL-Mevalonate-2-^{14}C (specific activity, 1.25 μc per μmole) was supplied by Tracerlab Inc., in the form of the dibenzylethylenediamine salt. It was converted into the potassium salt and tested in a soluble enzyme system from rat liver, where conversion in 25% yield to prenol pyrophosphates according to the procedure of Goodman and Popják (18) indicated its biological activity. Paper chromatography of the mevalonate-2-^{14}C in the isobutyric acid-NH_4OH-water (66:3:30) system (19) revealed the two major radioactive peaks at R_F 0.65 and 0.77 for the free acid and the lactone, respectively, but trace radioactive contaminants at R_F 0.93 and 0.52 were also detected. DL-Mevalonolactone-2-^{14}C (specific activity, 3.44 μc per μmole) was obtained from Nuclear Research Chemicals, Inc. The lactone was converted to the acid by incubation at 37° at pH 8.5 for 30 minutes; the excess alkali was then neutralized with hydrochloric acid. This preparation contained no discernible radioactive impurity on chromatography in the isobutyric acid-NH_4OH-water system.

Methods

Measurement of ^{3}H and ^{14}C

Simultaneous assay of ^{3}H and ^{14}C in a sample was performed with the use of the two-channel pulse height analysis of the

* Supported in part by Grant CA 03903, National Institutes of Health, United States Public Health Service.

† Investigator, Howard Hughes Medical Institute.

of fatty acid penetration into the erythrocyte. By the use of this method the rate at which the lower fatty acids, from formic through caprylic, penetrated beef and human erythrocyte was measured. It will be shown that the penetration rate was slowest for formic acid, increased to valeric acid and then again decreased in such a manner that heptylic and caprylic acid were found to penetrate more slowly than butyric acid. Lytic hemolysis studies were done with the Parpart densimeter ('35) and it was found that the results obtained in this way differed from those penetration data obtained by the spectrophotometric method.

METHODS

Since the work of Hüfner (1879) the absorption spectrum of oxyhemoglobin has been known and with instrumental improvement it has been measured with increased accuracy down to the present time (Hari, '17; Drabkin, '35; Rubinstein and Davidovich, '40). In a dilute suspension of erythrocytes oxyhemoglobin may be shown to change its absorption characteristics, as measured spectrophotometrically, by lowering the oxygen tension of the environment or by increasing the hydrogen ion concentration in the interior of the cells. This phenomenon by Barcroft and Murray ('22) provides a means of measuring by the use of a spectrophotometer the relative amount of acid which has penetrated a suspension of erythrocytes. Hartridge and Roughton in a series of studies ('23, '24) have shown that under their conditions of measurement the dissociation of oxyhemoglobin at lower pH values is nearly complete within 1/50 of a second. This information makes feasible the measurement of the rate of penetration of acid compounds into erythrocytes provided such penetration occurs more slowly than the limiting dissociation time of oxyhemoglobin.

A device was made for the rapid mixing of red cell suspension and acid solutions. A drawing of this appears in figure 1. It consists essentially of two reservoirs, 6 and 7

which empty through stopcocks, 8 and 9 either through the exit tubes 10 and 11 or through the master stopcock, 12, into a mixing chamber, 13. The mixing chamber, shown in the inset, is so constructed that complete mixing occurs within it and a homogeneous mixture runs into the observation tube, 14. The entire device was made of pyrex glass. For experi-

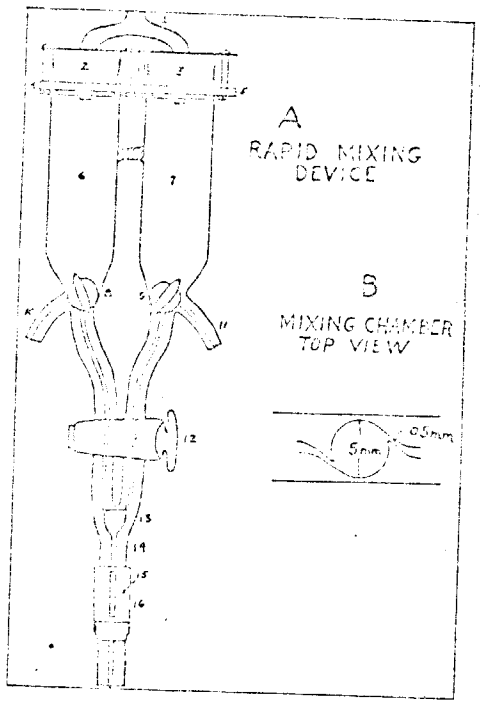


Fig. 1 Sketch of rapid-mixing device.

mental use the rapid mixing device was mounted above a Coleman, model 10S, spectrophotometer in such a manner that the observation tube passed vertically through the cuvette holder of the spectrophotometer. The monochromatic beam of light from the spectrophotometer then passed through the lumen of the observation tube. In order to focus the monochromatic beam to insure its passage through the lumen of

[illegible] tube, a glass collar (16 in fig. 1) [illegible] of optically flat glass [illegible] together with Dacrodhinky [illegible] and held in place on the observation tube by a piece of rubber tubing which [illegible] permitted changing its position as experiment required. A piece of thin brass shim containing a longitudinal slit [illegible] wide as the lumen of the observation tube and [illegible] as the beam of light from the spectrophotometer [illegible] inside the glass collar on the exit side of the light beam. When in use the glass collar was filled with glycerol which has approximately the same index of refraction as the pyrex glass.

When a red cell suspension is placed in one reservoir of [illegible] mixing device and a dilute solution of a lower fatty acid in the other, the two liquids will come together in the mixing chamber when the large stopcock is opened. From here they pass through the observation tube, traversing [illegible] the monochromatic beam of the spectrophotometer. It is apparent that the time which has elapsed from [illegible] in the mixing chamber until the mixture passes [illegible] center of the monochromatic beam represents the reaction time or the time in which the acid has had opportunity to enter the cell and bring about a dissociation of the [illegible] to a new equilibrium. Experimentally a measurement is made of the amount of light transmitted through the observation tube when the mixture is moving, i.e., a low reading, and when it is stopped, i.e., a higher reading. The difference between the two readings is taken as a relative measure of the acid which penetrated the cells in a given reaction time. The reaction time was varied by changing the mixing distance, i.e., the distance from the mixing chamber to the center of the monochromatic beam of the spectrophotometer, or by changing the speed of flow of the liquids leaving the reservoirs. The latter object was accomplished by fastening two clippers (2 and 3 in fig. 1) to the tops of the reservoirs and connecting the reservoirs to a compressed air source. By calibration the reaction times for a series of mixing distances

and pressures were determined. The following equations were used in the calculation of a given reaction time:

$$\text{L.F. (cm/sec)} = \frac{\text{V (cm}^3\text{/sec)}}{\text{A (cm}^2\text{)}} \qquad (1)$$

This equation states that the linear flow (L.F.) is equal to the volume (V) of liquid in milliliters per second passing through the observation tube divided by the area (A) of the lumen of the observation tube.

$$\text{R.T. (sec)} = \frac{\text{D (cm)}}{\text{L.F. (cm/sec)}} \qquad (2)$$

This second equation states that the reaction time (R.T.) measured in seconds is equal to the mixing distance (D) in centimeters divided by the L.F. obtained from the first equation.

Beef blood was obtained from one of the local slaughter houses and human blood was secured from laboratory personnel. Both types of blood were citrated at the time of collection to prevent clotting and were subsequently strained through sterile gauze to remove any clots which may have formed. Whole blood was used in the preparation of the red cell suspensions in concentrations of 1 to 1.5% blood in 1% NaCl solutions. Because of the initial acidity of the saline solutions a small amount of NaOH was added to bring the pH of the red cell suspension to approximately 7.4. Buffer solutions were not used because the method depended upon changing the pH of a red cell suspension from about 7.4 to 4.2. Further, one wished to study the penetration of the acid molecule and not the acid anion. The lower members of the saturated, monocarboxylic acid series from formic through caprylic acid were studied. Stock molar solutions of formic, acetic, propionic and butyric acids were prepared and appropriate dilutions made from these. Because of the relative insolubility of valeric, caproic, heptylic and caprylic acids, dilutions were prepared directly from the concentrated acid. Dilute acid solutions were prepared and used on the same day. Table 1 lists the acid concentrations used with the ap-

300

Packard scintillation spectrometer, model 500B, Series 314E. This permitted the counting of ^{14}C and ^{3}H in one channel at 7.8% and 20% efficiencies, respectively, and in the other at 52.3% and 0.0005%. All radioactivity measurements are presented as disintegrations per minute (d.p.m.), efficiencies being calculated for each set of measurements by the counting of appropriate toluene ^{14}C and ^{3}H standards. Samples were assayed in 5 ml of scintillation fluid containing 5 g of 2,5-diphenyloxazole and 100 mg of *p*-bis-2′-(5′ phenyloxazolyl)benzene per liter of toluene. In the case of very weakly radioactive samples, aliquots were chosen so as to give at least twice the background count, and were counted for a sufficient length of time to reduce the counting error to less than 10%.

Tissue Preparation and Incubation Conditions

For each experiment the ovary of a pregnant dairy cow was obtained 30 minutes post mortem and conveyed to the laboratory in ice-cold 0.9% sodium chloride. The corpus luteum was dissected and cut into 0.5-mm slices with a Stadie-Riggs hand microtome. The slices were distributed among the incubation vessels, each of which ultimately contained 300 to 750 mg of slices and 10 μc of the substrate under test in 5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4. Paired vessels were prepared for each substrate, one of each pair containing in addition 55 μg of LH.[1] The mevalonate-2-^{14}C tested in this experiment was the Tracerlab preparation (specific activity, 1.25 μc per μmole). For Experiment 2 exactly the same procedure was followed, except that each flask contained 0.988 to 1.419 g of slices, and 20 μg of LH were used for gonadotropin stimulation. This has been shown to be a saturating dosage (20). In this experiment the mevalonate-2-^{14}C used (specific activity, 3.14 μc per μmole) was obtained from Nuclear Research Chemicals, Inc. The incubations were carried out for 3 hours in a Dubnoff metabolic shaker at 37° in an atmosphere of 95% O_2 and 5% CO_2 and were terminated by freezing.

For each experiment 1 g of corpus luteum slices was frozen for subsequent analysis of the progesterone present in the tissue before incubation, thus permitting the calculation of the net quantity of progesterone formed during the incubation.

Extraction and Analysis of Lipids

The analytical procedure used is outlined in the flow sheet depicted in Fig. 1. To facilitate comparisons in reporting results, all data were corrected to 1.0 g, wet weight, of tissue. In addition, the progesterone values are corrected to 100% recovery by radioactive assay of a tracer amount of tritiated progesterone added at the start of the analysis and recovered in the eluate from the paper chromatograms in Method I, or from the thin layer chromatograms in Method II (Fig. 1). The values for squalene and sterols were not corrected for losses, but in preliminary experiments cholesterol-7-^{3}H recoveries were in the range of 85 to 95%.

Progesterone Analysis.—The details of Method I in Fig. 1 have previously been described (3). The only change introduced was the calculation of the recovery of progesterone by the use of the tritiated steroid, thus allowing a simultaneous assessment of progesterone-^{14}C and of the spectroscopically measurable progesterone produced from endogenous precursors during the course of the incubation. Method II (Fig. 1), used in the second experiment, involved the same initial ether extraction and subsequent countercurrent distribution between 90% methanol and ligroin as Method I. The polar fraction was then analyzed by two-dimensional thin layer chromatography as described by Armstrong, O'Brien, and Greep (21). The progesterone-containing areas on either paper or thin layer chromatograms were located by viewing in ultraviolet light. After elution, the progesterone concentration was obtained by measuring the optical density at 240 mμ in the Cary spectrophotometer, the Allen correction being used (22). An aliquot of the solution was counted, carrier progesterone was added, and the steroid was crystallized from ligroin to constant specific activity. In some cases the purity of the progesterone-^{14}C was checked by a second chromatographic separation in the ligroin-propylene glycol system.

Squalene and Sterols.—The aqueous residue obtained after initial ether extraction was combined with the nonpolar fraction of the countercurrent distribution and was saponified overnight at 60° in 2 N potassium hydroxide and 50% methanol. The nonsaponifiable fraction was then extracted three times with volumes of ether, and the ether extract was evaporated. The oily residue was then dissolved in 10 ml of 90% methanol and extracted five times with 30 ml of petroleum ether. The extract was washed once with 10 ml of water and was dried over magnesium sulfate. After filtration and evaporation to 2 ml, the extract was applied to a 2-g alumina column, 1 cm in diameter, prepared as described by Langdon and Bloch (23). Squalene and sterols were eluted as described (23), except that for elution of the sterol fraction, 20 ml of petroleum ether-benzene (9:1), were used to elute most of the yellow pigment present in the corpus luteum extracts. No radioactivity was detected in this colored fraction. The squalene fraction, eluted with petroleum ether, was proved to contain radiochemically pure squalene by the addition of 50 mg of carrier squalene to an aliquot, and the subsequent preparation of the hexahydrochloride and its crystallization of this derivative from acetone by the method of Langdon and Bloch (23).

After the addition of 2 mg of carrier cholesterol, digitonide precipitation by the method of Sperry (24) was performed on an aliquot of the sterol fraction. The sterols were released from the digitonide by pyridine treatment and ether extraction, and were assayed for radioactivity. The method was checked with cholesterol-7-^{3}H and resulted in the precipitation of more than 99.0% of the cholesterol.

Another aliquot of the sterol fraction was analyzed by thin layer chromatography on silica gel G plus Rhodamine 6G as described by Avigan, Goodman, and Steinberg (25). When benzene-ethyl acetate (5:1) is used as the solvent, the system is capable of separating C_{30} sterols (R_F, 0.69) from C_{27} sterols (R_F 0.49). On the basis of results reported by Goodman, Avigan, and Steinberg (26), the zone intermediate between C_{27} and C_{30} sterols was presumed to contain C_{28} and possibly C_{29} sterols. On each chromatogram lanosterol and cholesterol were run as reference substances in parallel with the mixtures to be analyzed. These were applied to the thin layer plate in bands 3 cm long. The fluorescent sterol zones were detected by viewing under ultraviolet light. Each zone was separately eluted by scraping powder off the plate and eluting with methylene chloride. After the initial chromatography, each individual zone was chromatographed in the same system and the radioactivity was assayed

[1] The abbreviation used is: LH, luteinizing hormone.

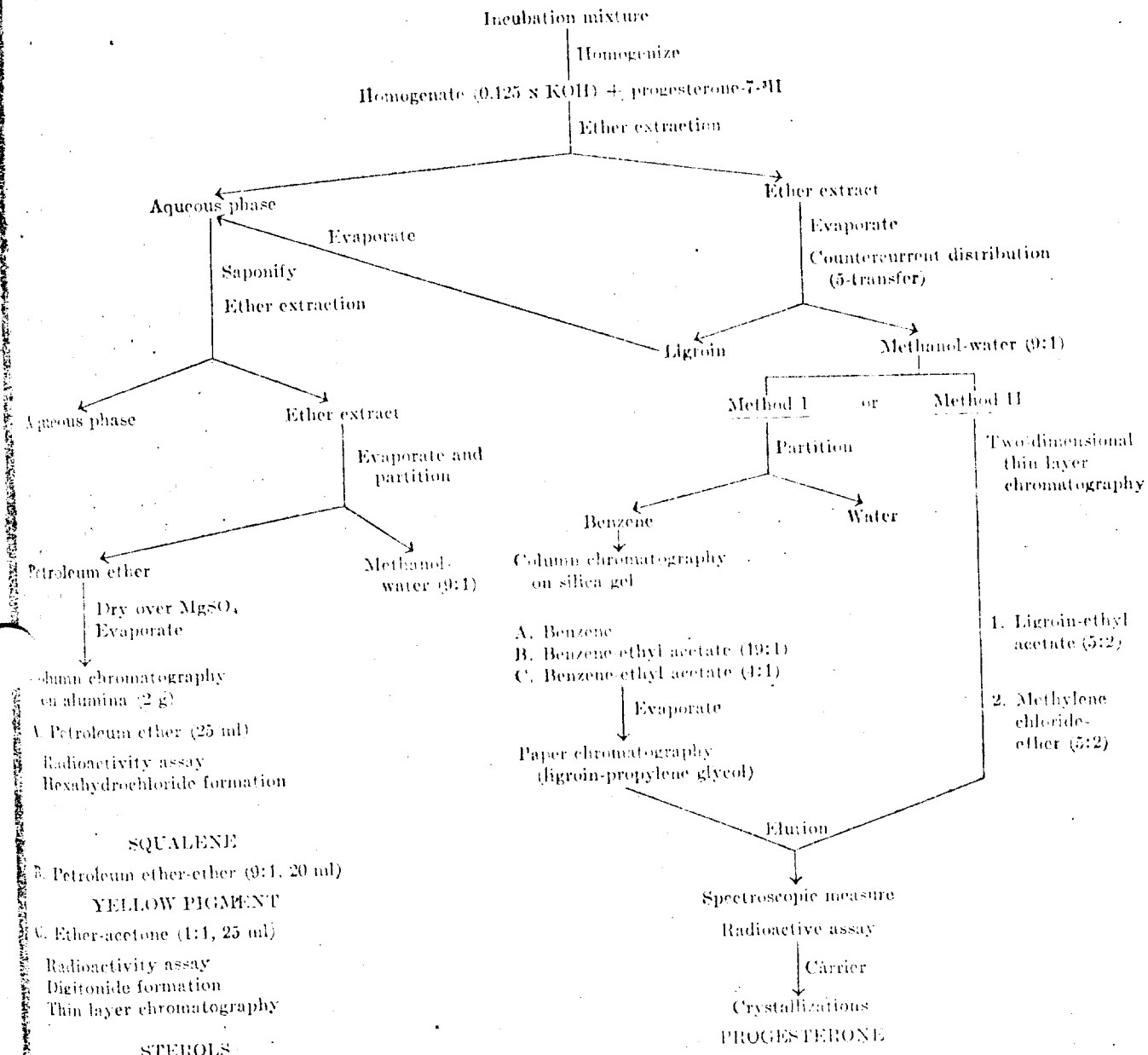


FIG. 1. Flow sheet of analysis for squalene, sterols, and progesterone.

RESULTS

In the experiments described, three compounds, acetate-1-^{14}C, mevalonate-2-^{14}C, and leucine-2-^{14}C, were tested as substrates for progesterone biosynthesis in slices of corpus luteum incubated in the absence and in the presence of exogenous pituitary LH. Purification of the radioactive steroid samples (Table I) showed that ^{14}C-labeled progesterone was formed from both acetate-1-^{14}C and mevalonate-2-^{14}C. The specific activity of the progesterone isolated from incubations containing leucine-2-^{14}C as possible substrate declined at each purification step. This resulted in the elimination of about 90% of the associated radioactivity; the remaining radioactivity was ascribed to contamination, and incorporation of ^{14}C from leucine was considered to be negligible.

The data of Table II show that the addition of LH to the incubation medium resulted in the increased utilization of both acetate-1-^{14}C and mevalonate-2-^{14}C for progesterone synthesis, concomitant with the increased formation of microgram amounts of the steroid. In both experiments, the incorporation of ^{14}C label from mevalonate-2-^{14}C, relative to that from acetate-1-^{14}C, was of a very much lower order. However, the increment in ^{14}C-labeling of progesterone brought about by the action of gonadotropin was almost the same for both substrates.

Table III shows the incorporation of radioactivity into squalene and sterols in Experiment 2. A partial characterization of the radioactive sterols was provided by digitonide precipitation and by thin layer chromatography. This table shows that the C_{27}, C_{28}, and C_{29} sterols synthesized by the bovine corpus luteum

TABLE I

Crystallization data on progesterone-^{14}C

Stage of analysis at which carrier progesterone was added is given in the text and in Fig. 1.

Substrate	Specific activity: Calculated after addition of carrier	First crystallization	Second crystallization	After paper chromatography
	d.p.m./mg	*d.p.m./mg*	*d.p.m./mg*	*d.p.m./mg*
Experiment 1				
1. Acetate-1-^{14}C	112,800	101,000	99,000	
2. Acetate-1-^{14}C	128,000	115,000	122,000	
3. Mevalonate-2-^{14}C	212	235	202	
4. Mevalonate-2-^{14}C	362	390	320	
5. Leucine-2-^{14}C	187	124	13	20*
6. Leucine-2-^{14}C	804	607	333	400*
Experiment 2				
1. Acetate-1-^{14}C	3,670	3,680	3,190	3,300†
2. Acetate-1-^{14}C	30,500	30,500	27,700	28,100
3. Mevalonate-2-^{14}C	216	178	196	207
4. Mevalonate-2-^{14}C	796	676	585	624

* In these two samples, constant specific activity was not achieved.

† Generally 70 to 90% of the progesterone-^{14}C recovered after purification was recrystallized. However, some of this sample was lost.

TABLE II

Effect of gonadotropin on progesterone synthesis

The incubation mixture consisted of 0.5-mm thick slices and 10 μc of the substrate under test in 5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4. LH was added as shown. The tissue weights in Experiment 1 varied from 300 to 750 mg and in Experiment 2 from 988 to 1,119 mg. The incubations were performed for 3 hours at 37° in an atmosphere of 95% O_2 and 5% CO_2.

Substrate	LH	Progesterone synthesis per g wet tissue		Increment in progesterone-^{14}C
	μs	*μs*	*d.p.m.*	%
Experiment 1				
1. Acetate-1-^{14}C, 20 μc per μmole		10	440,000	
2. Acetate-1-^{14}C, 20 μc per μmole	55	55	634,000	44
3. Mevalonate-2-^{14}C, 1.25 μc per μmole		42	1,530	
4. Mevalonate-2-^{14}C, 1.25 μc per μmole	55	60	2,380	56
Experiment 2				
1. Acetate-1-^{14}C, 20 μc per μmole		25	84,500	
2. Acetate-1-^{14}C, 20 μc per μmole	20	131	232,000	174
3. Mevalonate-2-^{14}C, 3.44 μc per μmole		23	1,250	
4. Mevalonate-2-^{14}C, 3.44 μc per μmole	20	112	3,620	191

all become labeled when either acetate-1-^{14}C or mevalonate-2-^{14}C are used as substrates. The C_{27} fraction is the most radioactive, in accord with the expected formation of cholesterol-^{14}C. The radiochemical purity of the squalene-^{14}C was established by hexahydrochloride formation following the addition of carrier squalene (Table IV).

DISCUSSION

The results expressed in Table II prove unambiguously that mevalonate-2-^{14}C is indeed used for progesterone biosynthesis by slices of bovine corpus luteum *in vitro*. Furthermore, in the presence of luteinizing hormone an increment in this utilization is obtained equal to that observed when acetate-1-^{14}C is used as a substrate for steroid synthesis.

A considerable synthesis of squalene and a number of sterol fractions are observed when mevalonate-2-^{14}C is utilized as a

TABLE III

Squalene-^{14}C and sterol-^{14}C formation by bovine corpus luteum

Incubation conditions given with Table II.

Experiment No.*	Squalene	Total sterols	Digitonide-precipitable sterols	Percentage distribution of ^{14}C-labeling in sterol fractions†		
				C_{26}	$C_{26} + C_{27}$	
	(d.p.m./g tissue) × 10⁻³			*%*		
2-1	214	2505	2500			
2-2	150	2520	2180	3.2	25.2	6
2-3	394	496	112	3.1	21.6	5
2-4	496	554	308			

* The substrates and amount of LH used in this experiment are shown in Experiment 2 of Table II.

† Determined by thin layer chromatography as described in "Methods."

TABLE IV

Crystallization data on Squalene-^{14}C hexahydrochloride

Substrate	Specific activity: Calculated after addition of carrier*	First crystallization	Second crystallization
	d.p.m./μmole		
Experiment 2			
1. Acetate-1-^{14}C	157	157	155
3. Mevalonate-2-^{14}C	249	236	256

* Calculated from weight of carrier squalene added to an aliquot of the eluate (A) from alumina column chromatography (Fig. 1).

TABLE V

Calculated amount, in millimicromoles, of squalene, digitonide-precipitable sterols, and progesterone formed from acetate-1-^{14}C and mevalonate-2-^{14}C

Incubation conditions are given with Table II. Basis of calculations is given in the text.

Substrate	LH	Amount of ^{14}C-labeled: Squalene	Sterol	Progesterone
	μs	*mμmoles*		
Experiment 1				
1. Acetate-1-^{14}C				0.992
2. Acetate-1-^{14}C	55			1.450
3. Mevalonate-2-^{14}C				0.183
4. Mevalonate-2-^{14}C	55			0.286
Experiment 2				
1. Acetate-1-^{14}C		0.396	4.70	0.191
2. Acetate-1-^{14}C	20	0.281	4.71	0.523
3. Mevalonate-2-^{14}C		9.44	11.88	0.060
4. Mevalonate-2-^{14}C	20	11.80	8.84	0.173

substrate (Tables III and IV). Recently similar findings have been reported by Salokangas, Rilling, and Samuels, who demonstrated the conversion of isopentenyl-1-^{14}C pyrophosphate to squalene and sterols in rat testis homogenates (27). They were, however, unable to demonstrate the utilization of the isoprenoid acid for steroid synthesis in this tissue.

As mentioned earlier, a number of other workers have failed to detect radioactive mevalonate incorporation into steroids in steroidogenic systems. A possible explanation of this difficulty is seen in the actual level of radioactive labeling of the steroid fraction observed in our experiments (Table II). It is about 100 times less when mevalonate-2-^{14}C is utilized as substrate than when the ^{14}C label is derived from acetate-1-^{14}C. However, direct comparison of radioactivity *per se* in a product derived from two different ^{14}C-labeled substrates may be meaningless unless the following factors are taken into account: (*a*) differences in specific activities of the substrates studied; (*b*) differences in the expected number of labeled positions in products synthesized from either a 2 carbon or a 6 carbon substrate, both of which are labeled in one position only; (*c*) unknown amounts of endogenous substrates in the tissue. Factor (*c*) cannot be calculated. If the assumption is made that squalene and sterols are formed in the corpus luteum by the same pathway as in liver, and that progesterone is formed from these precursors, the compounds studied would be labeled to a different extent, and in different positions, when formed from acetate-1-^{14}C or mevalonate-2-^{14}C. Partial degradation studies on cortisol biosynthesized from acetate-1-^{14}C by adrenal tissue support this view (28). With acetate-1-^{14}C as substrate, squalene, cholesterol, and progesterone would contain 12, 12, and 10 labeled positions, respectively. When biosynthesized from mevalonate-2-^{14}C, squalene would be labeled in 6 positions, cholesterol in 5, and progesterone in 3.

On the basis of this assumption, and knowing the specific activities of the acetate-1-^{14}C and mevalonate-2-^{14}C used, simplified calculations of the comparative utilization of these substrates for lipid synthesis by the corpus luteum may be made. Thus Table V presents the data of Tables II and III in terms of the number of millimicromoles of ^{14}C-labeled products formed from the radioactive substrates studied. (Isotope dilution due to endogenous substrates is not taken into account.) It is evident from these calculations that the utilization of mevalonate for squalene and sterol synthesis in the bovine corpus luteum appears to be comparable with that of acetate. However, the level of labeling of the progesterone derived from mevalonate-2-^{14}C is lower than might be expected compared either with the labeling of the sterol fraction formed from the same substrate, or with the degree of labeling of progesterone formed from acetate-1-^{14}C by slices of the same tissue. The reason for this apparent anomaly will be the subject of future investigation.

SUMMARY

The conversion of mevalonate-2-^{14}C as well as acetate-1-^{14}C to squalene, sterols, and progesterone by slices of bovine corpus luteum was established. The degree of ^{14}C-labeling of the products studied varied according to the substrate used. The utilization of both acetate-1-^{14}C and mevalonate-2-^{14}C for synthesis of progesterone-^{14}C was increased to a comparable degree by pituitary luteinizing hormone. The utilization of leucine-2-^{14}C for progesterone synthesis appeared to be negligible as compared with that of acetate and mevalonate.

Acknowledgments. The authors are indebted to Dr. Armstrong and his colleagues for details of their chromatographic procedure prior to publication.

REFERENCES

1. Brady, R. O., *J. Biol. Chem.*, **193**, 115 (1951).
2. Haynes, R., Savard, K., and Dorfman, R. I., *J. Biol. Chem.*, **207**, 925 (1954).
3. Mason, N. R., Marsh, J. M., and Savard, K., *J. Biol. Chem.*, **237**, 1801 (1962).
4. Savard, K., and Casey, P., *Endocrinology*, **74**, 509 (1964).
5. Rabinowitz, J. L., and Ragland, J. B., *Federation Proc.*, **17**, 293 (1958).
6. Savard, K., Goldweber, M., and Goldzieher, J. W., *Federation Proc.*, **19**, 169 (1960).
7. Roberts, K. S., and Webb, J. L., *Proc. Can. Federation Biol. Soc.*, **2**, 56 (1959).
8. Bryson, M. J., and Sweat, M. L., *Arch. Biochem. Biophys.*, **96**, 1 (1962).
9. Villee, D. B., Engel, L. L., Loring, J. M., and Villee, C. A., *Endocrinology*, **69**, 354 (1961).
10. Villee, D. B., Villee, C. A., Engel, L. L., and Talbot, N. B., *J. Clin. Endocrinol. and Metabolism*, **22**, 181 (1962).
11. Baer, T., Bonnet, J., and Neukomm, S., *Oncologia*, **13**, 289 (1960).
12. Mason, N. R., and Savard, K., *Endocrinology*, **75**, 215 (1964).
13. Tamaoki, B., and Pincus, G., *Endocrinology*, **69**, 527 (1961).
14. Hall, P. F., and Koritz, S. B., *Biochemistry*, **3**, 129 (1963).
15. Ichii, S., Forchielli, E., and Dorfman, R. I., *Steroids*, **2**, 631 (1963).
16. Hellig, H., and Savard, K., *Abstracts Sixth International Congress of Biochemistry 1964, IUB Vol. 32*, p. 576, Vol. 1.
17. Popják, G., and Cornforth, J. W., *Advances in Enzymol.*, **22**, 281 (1960).
18. Goodman, D. S., and Popják, G., *J. Lipid Research*, **1**, 286 (1960).
19. Waard, A. de., and Popják, G., *Biochem. J.*, **73**, 410 (1959).
20. Mason, N. R., and Savard, K., *Endocrinology*, **75**, 215 (1964).
21. Armstrong, D. T., O'Brien, J., and Grief, R. V., *Endocrinology*, **75**, 488 (1964).
22. Allen, W. M., *J. Clin. Endocrinol.*, **10**, 71 (1951).
23. Langdon, R. G., and Bloch, K., *J. Biol. Chem.*, **200**, 129 (1953).
24. Sperry, W. M., *J. Lipid Research*, **4**, 221 (1963).
25. Avigan, J., Goodman, D. S., and Steinberg, D., *J. Lipid Research*, **4**, 100 (1963).
26. Goodman, D. S., Avigan, J., and Steinberg, D., *J. Biol. Chem.*, **238**, 1287 (1963).
27. Salokangas, R. A., Rilling, H. C., and Samuels, L. T., *Biochemistry*, **3**, 833 (1964).
28. Casey, E., *Proceedings of Fifth International Congress of Biochemistry, Moscow, 1961, Vol. VII*, Pergamon Press, New York, 1963, p. 347.

Studies of Cholesterol Metabolism with C-14 Acetate Diabetic Patients and in Patients with Hypercholesterolemia

Metab. Clin. Exp. 11, 925-34, (1962)

By Alton R. Hennes, Margaret Z. Moore and Yanna F. Masters

Following the administration of C^{14} acetate to control subjects, stable adult diabetics and hypercholesterolemics, it was found that (1) diabetics did not differ from controls in incorporation of the carbon 14 into plasma cholesterol, (2) in some diabetics the chlorpropamide treatment is associated with a decrease in incorporation of C^{14} into cholesterol, and (3) hypercholesterolemics had specific activities for plasma cholesterol which did not differ markedly from the controls but the total findings may possibly be compatible with impaired removal of cholesterol from plasma.

THE USE OF C-14 ACETATE as a precursor to study endogenous cholesterol metabolism in man is fraught with considerable difficulty. The pool size of acetate and other cholesterol precursors is unknown. The pool size of cholesterol in the tissues is also unknown. Further, the population groups studied are heterogeneous, as is their previous dietary intake. In spite of these impediments, the use of C-14 labeled precursors may be helpful in allowing study of rates of removal of a cholesterol molecule which is in all likelihood handled exactly like any other endogenously synthesized cholesterol molecule. Further, it is possible that by determining total amounts of incorporation of a labeled precursor into plasma cholesterol along with the turnover rates, information can be obtained which will aid in determining rather grossly whether the plasma level of cholesterol is elevated because of increased incorporation of precursors into cholesterol or because of decreased removal of cholesterol.

In this series of studies, incorporation of C-14 from 1-C-14 acetate into free and esterified cholesterol has been studied in a group of subjects with diabetes and in subjects with idiopathic hypercholesterolemia.

Procedure

All control, diabetic, and hypercholesterolemic subjects were hospitalized and received a diet consisting of 250 Gm. of carbohydrates, 80 Gm. of protein, and 2200 to 2400 calories, depending on their weight. All subjects without diabetes had a normal 3 hour glucose tolerance test. All subjects were given 100 μc of 1-C-14 acetate with 1 mM of carrier sodium acetate in a single rapid intravenous injection. Samples were obtained in the fasting state at 1, 2, 3, 4, 6, and 8 hours after the injection. A further sample was obtained at 24 hours in the fasting state. Free and esterified cholesterol were separated by silicic acid column chromatography by the method of Barron and Hanahan.[1] Cholesterol was determined on an eluate from the column by the method of Carr and Drekter.[2] The esterified cholesterol fraction was saponified prior to counting to remove radioactive ester cholesterol fatty acids. The free cholesterol fraction was also saponified prior to counting to remove any contaminating mono- and diglycerides and nonesterified fatty acids. Radioactivity was then determined in each fraction with a tricarb liquid scintillation spectrometer.

The diabetic subjects were all typical stable adult, or insulin-independent diabetics. Several were studied prior to any treatment. Several others were studied while under therapy with chlorpropamide. Two diabetics were studied before and after treatment with chlorpropamide. One had previously been well controlled with insulin, with perfectly normal fasting blood sugar. The other was studied prior to any treatment. One obese subject with an abnormal glucose tolerance test also had elevated triglycerides and hypercholesterolemia.

Five subjects with hypercholesterolemia, without diabetes, were studied. Three of the five had had previous myocardial infarctions. Two of these three patients had, in addition to hypercholesterolemia, moderate elevation of triglycerides in the range of 150 to 300 mg. per cent. One subject with hypercholesterolemia secondary to biliary obstruction due to stone was also studied. Clinical data on control and experimental subjects are given in tables 1, 2, and 3.

Results

It can be seen in table 4 that there is a wide range of specific activities of free cholesterol in the various groups of subjects. The wide range of specific activities in studies of this type has been previously noted by Gould et al.[3] As also noted by Gould et al.,[3] the peak specific activity in free cholesterol is reached between 2 and 4 hours after administration of acetate. The per cent

From the Department of Medicine, Wayne State University College of Medicine, Detroit and Veterans Administration Hospital, Oklahoma City, Oklahoma.

This work was supported in part by N.I.H. Grant A-3191 and a grant by Charles Pfizer and Company.

Received for publication May 8, 1962.

Table 1.—"Control" Subjects

Subject	Age	Weight (Kg.)	Diagnosis	Cholesterol (mg. %)
J. E.	46	75	Convalescent from viral pneumonitis	206
J. B.	62	77.3	Addison's disease, on maintenance therapy	213
J. S.	63	61	Slight pulmonary fibrosis	267
R. McC.	54	52.7	Hemorrhoids	19[illegible]
W. H.	48	70	No disease	16[illegible]
L. S.	49	71.5	Treated pernicious anemia	36[illegible]
N. B.	65	77.3	Addison's disease, on maintenance therapy	20[illegible]

Table 2.—Diabetic Subjects

Subject	Age	Weight (Kg.)	Treatment	Complications	FBS	Cholesterol (mg. %)
W. Sh.$_1$	52	87.5	Insulin	None	82	240
W. Sh.$_2$	52	86	Chlorpropamide	None	68	246
W. St.$_1$	64	79	None	None	122	266
W. St.$_2$	64	78.6	Chlorpropamide	None	81	266
C. C.	68	91	Chlorpropamide	None	70	252
W. B.$_1$	66	68	Chlorpropamide for 2 wks.	Mild peripheral neuropathy	130	332
W. B.$_2$	66	66	Chlorpropamide for 5 mo.	Mild peripheral neuropathy	90	322
H. K.	63	67	None	Myocardial infarction	245	322
R. A.	39	95.5	None	None	140	315
C. F.	46	84.6	None	None	110*	460

*Normal: 80–120. All other subjects, normal: 60–90.

Table 3.—*Patients with Idiopathic Hypercholesterolemia*

Subject	Age	Weight (Kg.)	Complications	Total cholesterol (mg. %)
A. R.	39	70	Myocardial infarction	432
L. W.	41	70.4	Myocardial infarction	613
I. H.	54	77.5	Myocardial infarction	433
W. B.	48	70	None	420
S. O.	54	103	Xanthelasma	540

of C^{14} incorporated into free cholesterol which is determined by the peak specific activity, level of cholesterol recovered from the silicic acid column and estimated plasma volume, also has a wide range in the various groups of subjects. In figure 1, it can be seen that the curves for incorporation and decline of radioactivity into free cholesterol are generally quite similar in shape in the three groups of subjects. The pattern of incorporation of radioactivity into esterified cholesterol also appears to be quite similar in the three groups. This general pattern of incorporation is very similar to that found by Eckles et al. in dogs.[4] As previously noted by Hellman, Rosenfeld, and Gallagher,[5] the free cholesterol data when plotted on semilogarithmic paper usually yield two straight lines, indicating two turnover rates during the time period studied.

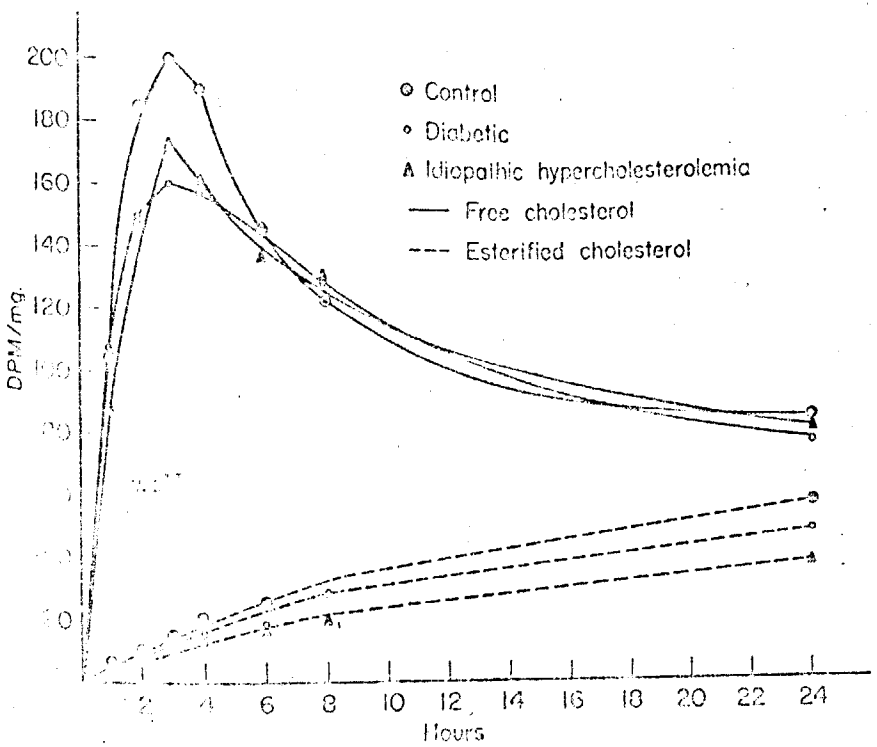


Fig. 1.—Specific activity of plasma-free and esterified cholesterol in representative control and experimental subjects.

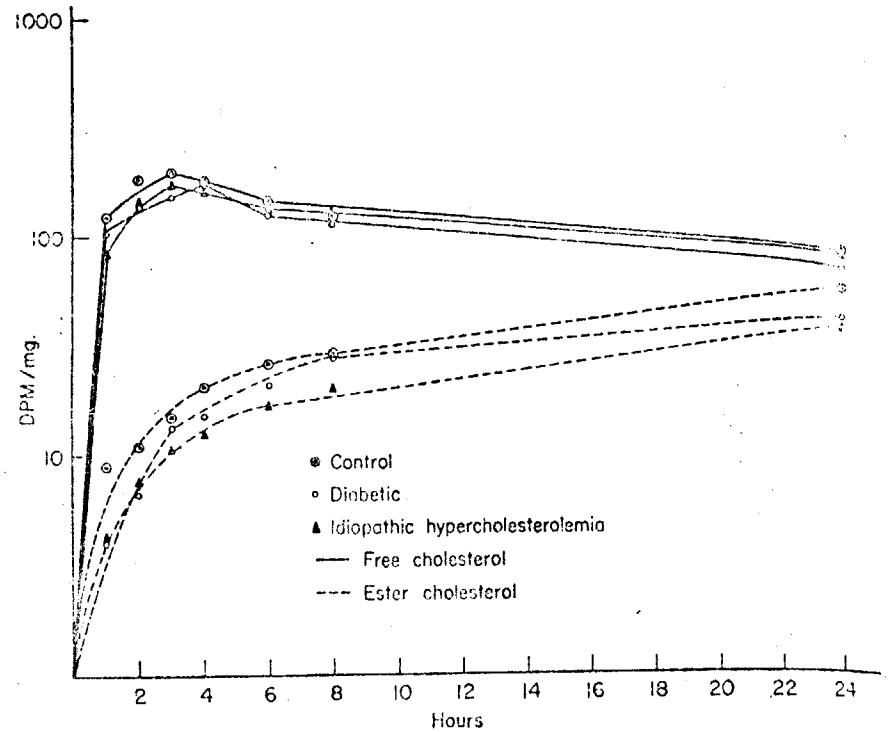


Fig. 2.—Semilogarithmic plot of specific activity data shown in figure 1.

The slope of the first line is considerably steeper than the second. These two early phases of exponential decline in specific activity can be seen in figure 2. As further noted by Hellman et al.,[5] the two early rapid turnover rates of plasma-free cholesterol seem to be due largely to endogenous recycling of free cholesterol into esterified cholesterol, since if the total amount of radioactivity present in free and ester cholesterol is calculated at the peak of incorporation into free cholesterol, an almost similar amount of radioactivity is present when free and cholesterol ester specific activities intersect. Unfortunately, most of these experiments were not carried out long enough to determine the exact specific activity at the crossover of free and ester cholesterol. However, in those experiments where the crossover occurred at or prior to 24 hours, most of the radioactivity present in free and ester cholesterol at earlier time periods could still be accounted for in plasma cholesterol.

It can be seen in figure 3 that there is quite a good correlation between the specific activity of ester cholesterol and that of free cholesterol with an r value of 0.85 for the entire series. In figures 1 and 2, it can be seen that the pattern of incorporation of activity into ester cholesterol has in general two phases, an early rather rapid and a later slower phase. These phases can be made to approximate fairly grossly two straight lines with differing slopes or two gently sloping curves. It seems likely from the two differing slopes

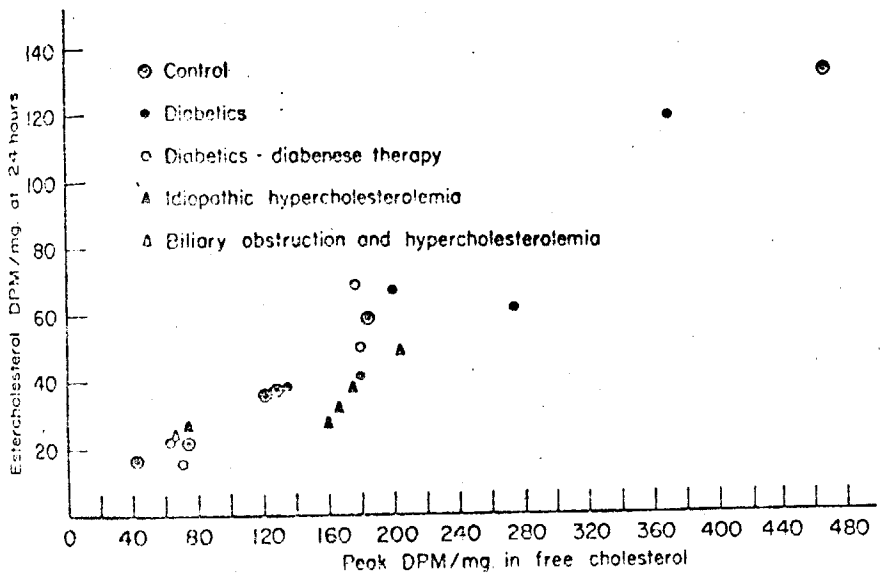


Fig. 3.—Relationship between peak specific activity of free cholesterol and 24 hour specific activity of ester cholesterol.

of incorporation of activity into ester cholesterol that two different precursor pools exist in the liver for esterification of ester cholesterol and that these reflect the two logarithmic processes of disposal of free cholesterol described above. It seems most logical that the pattern of incorporation of radioactivity into ester cholesterol should represent two different curves because of gradually and continually decreasing specific activities in the free cholesterol pools which serve as the precursors for esterified cholesterol.

The control and diabetic subjects can be seen to have quite a similar range of incorporation of radioactivity into plasma-free cholesterol and similar specific activities in plasma-free cholesterol. Incorporation of radioactivity into esterified cholesterol is also quite similar and follows the usual precursor product relationship for free and esterified cholesterol (table 4). However, in one mild, obese diabetic with only an abnormal glucose tolerance test, but with elevated triglycerides and hypercholesterolemia, incorporation of radioactivity into cholesterol is considerably greater than most of the controls and the other diabetic subjects.

Two diabetic patients were studied before and after treatment with chlorpropamide. One diabetic had been previously well controlled with insulin. One had been studied prior to any therapy and had moderately elevated fasting blood sugar. Excellent control of diabetes with chlorpropamide was associated in both cases with a striking decrease in incorporation of C^{14} into free and esterified cholesterol (fig. 4). The striking decrease in incorporation of C^{14} occurred without definite change in level of plasma cholesterol. It seems likely therefore, that the processes involved in degradation and synthesis of cholesterol were proportionately slowed, resulting in no net change in level of

Table 4.—*Specific Activities*

Patients	1 Hour Free*	1 Hour Ester*	2 Hours Free*	2 Hours Ester*	3 Hours Free*	3 Hours Ester*	4 Hours Free*	4 Hours Ester*	6 Hours Free*	6 Hours Ester*	8 Hours Free*	8 Hours Ester*	24 Hours Free*	24 Hours Ester*	Maximum % incorporation in plasma-free cholesterol
Controls															
J. E.	125	9	184	11	199	15	180	21	148	26	125	28	85	58	0.19
J. B.	84	7	97	11	—	—	121	15	79	17	44	20	48	36	0.12
J. S.	89	6	129	9	117	13	98	14	85	20	82	23	76	37	0.07
R. McC.	40	2	69	5	73	6	69	7	54	8	16	10	9	22	0.04
W. H.	31	0	42	1	41	2.3	41	2.5	30	4	28	5.4	16	9	0.024
L. S.	391	24	826	36	939	51	929	73	—	93	590	111	—	—	1.0
N. B.	180	16	358	41	466	49	445	61	372	78	324	94	176	133	0.39
Diabetics															
W. Sh_1	102	4	140	7	156	13	178	15	128	22	115	28	71	42	0.097
W. Sh_2	34	2	59	5	62	8	62	9	51	11	41	9	34	22	0.03
W. St_1	108	5	164	9	184	13	174	16	153	19	123	29	83	38	0.15
W. St_2	38	0	68	2	64	4	70	5.6	63	6.4	52	10	29	16	0.05
C. C.	98	7	178	14	153	18	148	20	116	33	111	38	81	52	0.14
W. B_1	138	6	196	13	205	18	195	20	150	27	142	33	45	49	0.22
W. B_2	87	6	—	9	160	10	168	15	153	19	160	28	108	64	0.17
H. K.	114	9	186	21	200	28	170	30	151	40	143	48	88	67	0.07
R. A.	255	17	338	31	371	43	341	60	311	67	285	73	168	123	0.41
C. F.	154	6.4	201	11	276	16	217	24	194	32	180	37	103	61	0.73
Idiopathic hypercholesterolemia															
A. R.	88	4	147	8	173	11	162	13	136	17	131	20	82	33	0.18
L. W.	99	4	163	8	—	9	163	12	138	15	125	18	83	34	0.40
I. H.	72	4	71	5	71	6	74	8.6	58	8.9	52	10	37	18	0.11
W. B.	27	2	18	4	70	8	74	10	64	13	55	14	37	23	0.09
S. O.	83	2	140	5	159	7	142	9	130	12	115	15	71	27	0.28
Biliary obstruction with hypercholesterolemia															
R. L. S.	43	2.4	65	5	61	6	58	8	52	10	[illegible]	[illegible]	[illegible]	22	0.076

*DPM/mg.

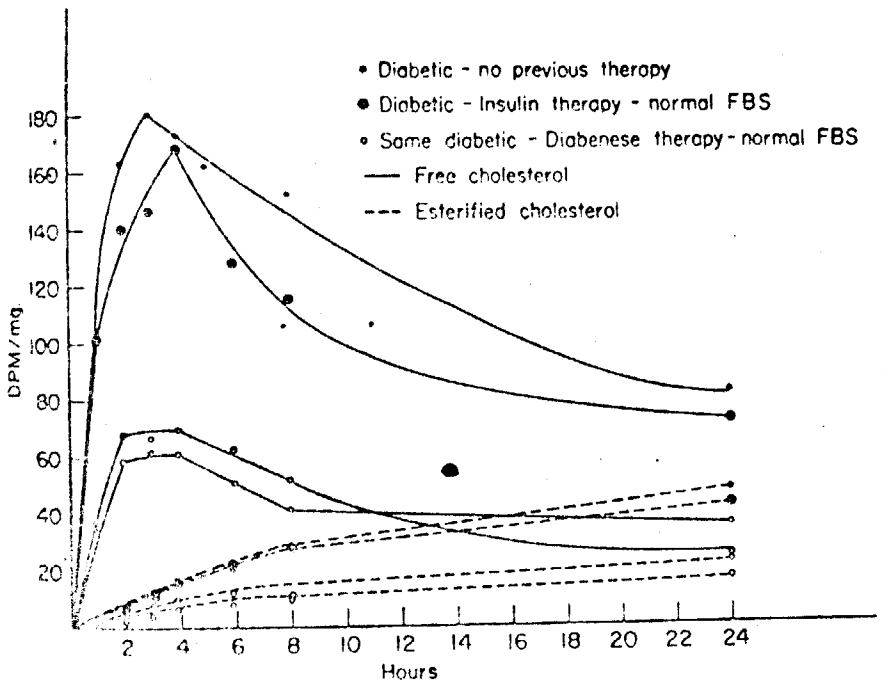


Fig. 4.—Effect of chlorpropamide therapy on specific activity of free and esterified cholesterol.

plasma cholesterol. Three other studies have been done in patients treated with chlorpropamide. However, control studies were not done prior to treatment.

Patients with idiopathic hypercholesterolemia appear in general to incorporate amounts of C-14 into plasma cholesterol similar to the control subjects (fig. 4). However, it can be seen in figure 4 that controls appear to have quite a wide range of incorporation of radioactivity into plasma-free cholesterol with quite a limited level of free cholesterol in plasma. On the other hand patients with hypercholesterolemia appear to have a higher level of plasma cholesterol at a given level of incorporation of radioactivity into cholesterol. In this respect, they are quite similar to a patient with biliary obstruction and hypercholesterolemia who was found to have a high level of plasma-free cholesterol with a relatively low total incorporation of radioactivity into cholesterol (fig. 5). The ratio of specific activity of plasma-free cholesterol at 24 hours to peak specific activity of plasma-free cholesterol (table 5) is generally higher in hypercholesterolemic subjects than in the control group. The fractional turnover rates have been determined for the two early phases of cholesterol removal and appear to be somewhat decreased in subjects with hypercholesterolemia. However, enough data are not available to determine the turnover rates with sufficient accuracy and minor deviations in the available data result in rather striking changes in

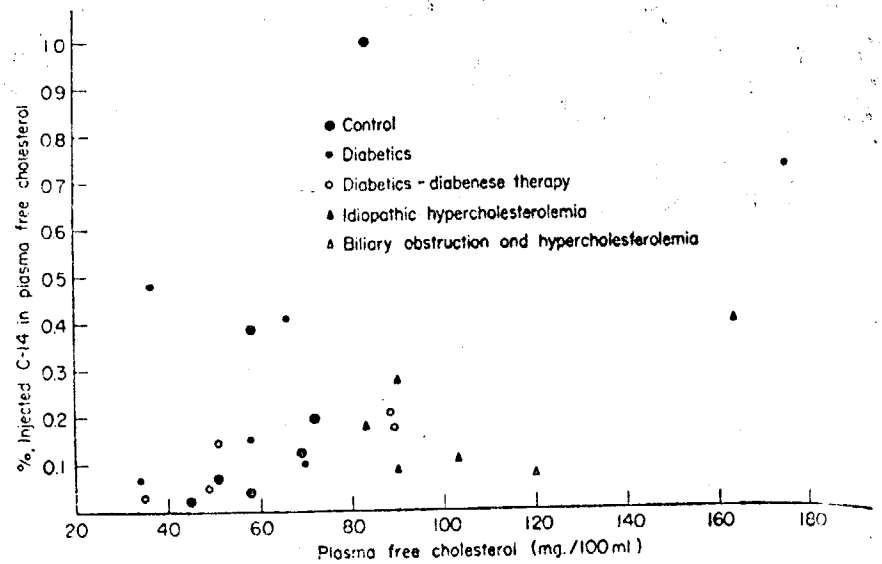


Fig. 5.—Relationship between incorporation of C^{14} into free cholesterol and level of plasma-free cholesterol.

turnover rates. Therefore, the exact determination of these turnover rates must await further, more complete studies.

Discussion

It can be seen from these data that control and stable adult diabetic subjects, whether treated or untreated, appear to incorporate similar amounts of C^{-14} from 1-C^{-14} acetate into plasma cholesterol. This finding is similar to that previously reported in a group of mild, well controlled, insulin-treated diabetic subjects.[6] It may be of some interest, however, that the one diabetic patient with only an abnormal glucose tolerance test, but with hypertriglyceridemia and hypercholesterolemia, showed an increase in incorporation of radioactivity into cholesterol in comparison to all the other patients studied, except for one control subject. It may be of interest in relation to this finding that insulin has been found to increase cholesterol synthesis in perfused rat livers[7] and that elevated levels of insulin have been found in some early, mild, obese diabetics.[8]

Control of diabetes with chloropropamide was found to be associated in some cases with a decreased incorporation of C^{-14} into plasma-free and esterified cholesterol. Since the cholesterol level appears to be unchanged, processes involved in degradation must be proportionately slowed, resulting in slowed cholesterol turnover.

Five subjects with idiopathic hypercholesterolemia were found to incorporate amounts of C^{-14} into cholesterol similar to that in control and diabetic subjects. However, at a given level of incorporation, the level of plasma-free

Table 5.—*Ratio of Specific Activity of Plasma-free Cholesterol at 24 Hours to Peak Specific Activity*

Patient	Controls	Diabetics	Idiopathic hypercholesterolemia	Biliary obstruction
J. E.	0.42			
J. B.	0.39			
J. S.	0.58			
R. McC.	0.22			
W. H.	0.38			
N. B.	0.36			
W. Sh.$_1$		0.40		
W. Sh.$_2$		0.52		
W. St.$_1$		0.45		
W. St.$_2$		0.41		
C. C.		0.46		
W. B.$_1$		0.21		
W. B.$_2$		0.25		
H. K.		0.38		
R. A.		0.45		
C. F.		0.38		
A. R.			0.47	
L. W.			0.46	
I. H.			0.50	
W. B.			0.50	
S. O.			0.44	
R. S.				0.58
Mean	0.39	0.39	0.47	0.58

cholesterol appeared to be generally much higher in subjects with hypercholesterolemia than in control and diabetic subjects, with the exception of a diabetic subject with hypercholesterolemia. This finding suggests that in subjects with idiopathic hypercholesterolemia processes involved in degradation of cholesterol may be at fault rather than synthetic processes. Some support for this concept was given by the generally higher ratio of specific activity at 24 hours as compared to peak specific activity in hypercholesterolemia as compared to the control subjects.

REFERENCES

1. Bar[illegible], E. J., and Hanahan, D. J.: Observations on the silicic acid chromatography of the neutral lipids of rat liver, [illegible] of liver, and yeast. J. Biol. Chem. [illegible]:493, 1958.
2. Carr, J. J., and Drekter, I. J.: Simplified rapid technic for extraction and determination of serum cholesterol without saponification. Clin. Chem. 2:353, 1956.
3. Gould, R. G., LeRoy, G. V., Okita, G. T., Kabara, J. J., Keegan, P., and Bergenstal, D. M.: The use of C-14 labeled acetate to study cholesterol metabolism in man. J. Lab. & Clin. Med. 46:372, 1955.
4. Eckles, N. E., Taylor, C. B., Campbell, D. J., Gould, R. G., Kelly, F. B., Davis, C. B., and Latenser, J.: The origin of plasma cholesterol and the rates of equilibration of liver, plasma, and erythrocyte cholesterol. J. Lab. & Clin. Med. 46:359, 1955.
5. Hellman, L., Rosenfeld, R. S., and Gallagher, T. F.: Cholesterol synthesis from C-14 acetate in man. J. Clin. Invest. 33:142, 1954.

6. Hennes, A. R., and Redding, T. W.: Defective synthesis of triglyceride fatty acids from 1-C-14 acetate in the well controlled stable adult diabetic. Diabetes 10:85, 1961.
7. Williams, W. R., Hill, R., and Chaikoff, I. L.: Portal venous injection of insulin in the diabetic rat: Time of induction of changes in hepatic lipogenesis, cholesterogenesis, and glycogenesis. J. Lipid Res. 1:236, 1960.
8. Randle, P. J.-quoted by Vallance-Owen, J., and Lilley, M. D.: Insulin antagonism in the plasma of obese diabetics and prediabetics. Lancet 1:806, 1961.

Allen R. Hennes, M.D., Associate Professor of Medicine and Chief, Section of Endocrinology, Wayne State University College of Medicine, Detroit, Michigan.

Margaret Z. Moore, B.S., Research Technician (Biochemistry), Veterans Administration Hospital, Oklahoma City, Oklahoma.

Yanna F. Masters, M.S., Research Technician (Biochemistry), Veterans Administration Hospital, Oklahoma City, Oklahoma.

German Translation

Acetic Acid as End Product of Aerobic Cancer Metabolism

Dieter Hepp, Eva Prusse, Hans Weiss* and Otto Wieland** (Clinical Chemistry and Biochemistry Laboratory of the Second Medical University Clinic, Munich)

Source: Biochem. Zeitschr. 344:87-102 (1966)

Summary

In four different strains of ascites tumors and in one solid hepatocarcinoma, acetate was observed to constitute an end-product of aerobic metabolism of pyruvate and palmitate. According to enzymatic studies with soluble tumor extracts, there is a striking decrease in the activity of acetate thiokinase as compared to liver tissue which does not form acetate. On the other hand, the activity of the acetyl-CoA splitting enzyme(s) is undiminished in tumor extracts. It is assumed that net formation of acetate in tumors is the result of an imbalance of the acetate activating enzyme and the acetyl-CoA splitting enzyme(s). The implications of the permanent loss of respiratory energy caused by this defect in the tumor enzyme pattern with regard to the development of aerobic glycolysis of malignant tissues is dissussed.

During studies on acetic acid metabolism of animal tissues (18), it was found that strikingly large amounts of acetate are produced by mouse ascites

* This study contains excerpts from the dissertation thesis of H. Weiss, Munich (1964/65).

** Reported in condensed form at the "Fourth International Symposium on Regulation of Enzyme Activity in Normal and Neoplastic Tissues", Indianapolis, 4-5 Oct. 1965 and at the Fall meeting of the German Society of Physiological Chemistry, Berlin, 6-9 Oct. 1965.

tumor cells *in vitro*. We have investigated this phenomenon further with the purpose of clarifying the mechanisms lying at the basis of the increased acetate formation by tumor cells and in order to judge whether we are dealing here with an anomaly of tumor metabolism of broad interest. The results of these studies are reported here.

Methods

Tumors. The ascites tumors studied here are given the following designations in this article (see note):

a. MAT I: Mouse ascites tumor, diploid.

b. MAT II: Mouse ascites tumor, tetraploid. The strains were cultivated at the Pathological Institute of Munich University (45).

c. RAT I: Rat Yoshida ascites sarcoma, strain RY 144 (45).

d. RAT II: Rat Zajdela ascites hepatoma (46).

(Note: Tumors a, b, and c were graciously supplied by Prof. H. Wrba, Pathological Institute of Munich University, and tumor d by Prof. G. Ruhenstroth-Bauer, Max Planck Biochemistry Institute, Munich).

MAT I, the most extensively studied tumor was further cultivated in 34 passages on NMRI mice (supplied by W. Gassner, Munich-Ottobrunn) and showed no significant differences in cell numbers and cell volume from one passage to another.

Preparation of tumor cells. Ascites was collected 7-11 days after transplantation in the usual way by puncture of the abdominal cavity through the open peritoneum with a Rekord syringe and immediately washed with ice-cold Ringer solution in a centrifuge at 4°C and about 1000 rpm. Samples tinged with blood were rejected. In the case of RAT II, most of the erythro-

cytes were removed by brief, low-rotational centrifuging.

For incubation tests with intact cells, harvested tumor cells obtained immediately after the third washing were used. For the preparation of protein extracts, portion of the washed and compacted cells was freeze-dried or preserved at -15°C in a deep-freeze compartment.

Test with intact tumor cells. In general, fresh cells were incubated in small Erlenmeyer flasks (25 ml volume) or in Warburg containers. For the isotope tests, we used containers described elsewhere (42), which were modified in such a manner that the alkali-containing glass insert could be removed and immersed, together with its content, into the measuring vessel with the scintillation liquid. The amounts of tissue used were measured either by weighing or by pipeting the uniformly distributed cell suspensions (magnetic stirrer). All incubation tests were performed under shaking in a constant-temperature water bath at 37°C.

For protein determination by the Giuret method (2), the packed cells were weighed in a glass homogenizer and homogenized in a definite volume. An aliquot of the total homogenate was used for the determination.

The same procedure was used on liver and tumor thin sections.

Tests with tumor extracts. In the preparation of protein-containing crude extracts for enzyme studies, the washed tumor cells were at first frozen in liquid air and subjected to a sharp extraction (solubilization) in a Potter glass homogenizer or in a knife homogenizer (supplied by MSE Ltd., London W.W.I England) with the appropriate buffer solution in a 1:3 ratio. Extraction of the lyophillizates was done in the same manner (buffer to dry weight ratio = 25:1 vol/wt.). The crude extracts were centrifuged in a Spinco centrifuge at 105,000 rpm. The protein content of the extracts

was determined by the biuret method (2).

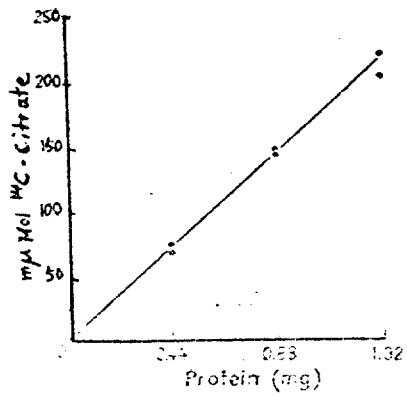
Acetate determination. Acetic acid was determined by the method of Lundquist (24). In this method, acetic acid was first separated by Conway microdiffusion from protein-free samples and then enzymatically determined by the sulfanilamide acetylation test of Soodak and Lipmann (38). The centrifuged incubation batches, stopped with 1 vol. of 6% perchloric acid, were adjusted to pH 3 (ice bath!) with 6 N NaOH and 0.5 M Na-citrate buffer (pH 3) according to the original article (24), and 1 ml of this solution was used in the microdiffusion procedure. For further details, see the original article (24) and an earlier publication (18). Each series of determinations was controlled (checked) with a standard acetate solution by means of a 6-point calibration curve.

Enzyme determinations. (a). Acetate thiokinase. The hydroxamic acid method (22) was found to be too sensitive for the determination of acetate thiokinase in tumor extracts. Therefore, an isotope method was worked out, which is based on the following reactions:

$$1\text{-}^{14}\text{C-Acetate} + \text{ATP} + \text{CoA} \xrightleftharpoons{\text{Ac-thiokinase}} 1\text{-}^{14}\text{C-Acetyl-CoA} + \text{AMP} + \text{PP} \quad (1)$$

$$1\text{-}^{14}\text{C-Acetyl-CoA} + \text{Oxalacetate} + H_2O \xrightleftharpoons{\text{Citratesynthase}} 1\text{-}^{14}\text{C-Citrate} + \text{CoA} \quad (2)$$

The 1-^{14}C-acetyl-CoA, formed in reaction # 1 by enzymatic activation of radioactively labeled acetate, is converted to labeled citrate with citrate synthase and oxalacetate in the auxiliary reaction # 2. The citrate can be quantitatively isolated from the reaction batch by precipitation as silver salt and measured. In this procedure, the amount of acetate incorporated is proportional, within a wide range, to the amount of acetate thiokinase used (Fig. 1). The time dependence of the test is shown in Fig. 2.



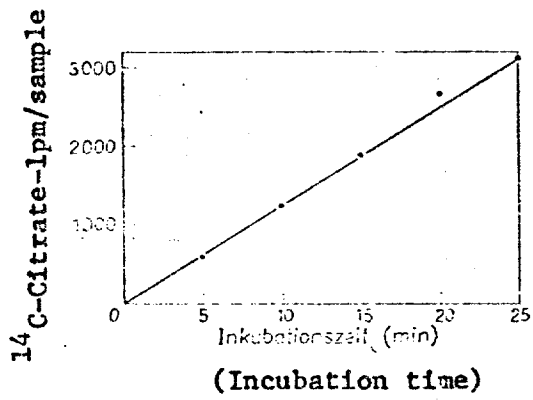


Fig. 1. **Fig. 2.**

Fig. 1. Isotope test for the determination of acetate thiokinase. Calibrating plot with increasing amounts of a particle-free mouse liver extract. For test procedure, see method.

Fig. 2. Isotope test for the determination of acetate thiokinase. Dependence of ^{14}C-citrate formation on incubation time. Amount of protein used (particle-free tumor cell extract-MAT I): 4.1 mg per batch. For further test procedure, see method.

Test batch. The following amounts of compounds were pipeted successively into 13 x 98 mm centrifuge tubes: TRIS-HNO_3 buffer, pH 7.4, 60 μ Mol, glutathione-SH 10 μ Mol, ATP 5 μ Mol, $MgSO_4$ 5 μ Mol, CoA-SH 0.4 μ Mol, oxalacetate 18 μ Mol, 1-^{14}C-acetate 5 μ Mol (corresponding to about 900,000 I.p.m.), crystalline citrate synthase 20 μg, crude extract (particle-free, 105,000 rpm supernatant) corresponding to 0.4-6 mg protein. Final volume 1ml, incubation 20 min at 37°C. The batches are stopped by adding 0.2 ml 0.5 M citric acid and boiling for 5 min on the water bath, adjusted to pH 5 (indicator paper) with 2N KOH after cooling, and 2 mg $AgNO_3$ reagent was added (dissolve 5 g $AgNO_3$ in about 80 ml water, add 4 ml 0.5 M TRIS-solution and 0.15 ml 2N HNO_3, and fill up with water to 100 ml). After addition of 4 ml acetone-alcohol (1:1) and standing for 10 min in the dark, precipitation is complete. The precipitate is washed 3 times in the centrifuge with a 4 M acetone-alcohol-acetic acid mixture (10:10:1 ratio) and dissolved in

1 ml 0.1N HNO_3. After dilution with 1 ml H_2O, excess silver is removed by precipitation with 0.1 ml 5N HCl and centrifuging. From the supernatant, 0.5 ml was pipeted into a small screw glass container for the Packard scintillation counter, and evaporated to dryness at 80°C after addition of 0.2 ml 4 M acetic acid. For counting, the residue was first dissolved with 0.1 ml 0.1N NaOH and then filled up with 10 ml of Bray's solution (5). A batch stopped at time 0 and treated further as described above served as a blank.

In enzyme determinations, the counter efficiencies were at the same level as with aqueous ^{14}C-acetate standard solutions, so that a correction was not necessary (Note: all radioactivity measurements were performed with a Tri-Carb scintillation counter, model 3003, made by the Packard firm, LaGrange, Illinois). The acetate thiokenase activity is given in international milliunits (mμ Mol acetate incorporated per min per mg protein). A comparison of the isotope test with the hydroxamic acid method (22,30), carried out on liver extracts, showed that the values obtained agreed with each other.

(b). Acetyl-CoA-deacylase. For the determination of acetyl-CoA-deacylase, the discoloration of dichlorophenolindophenol by CoA-SH liberated during cleavage was measured, in accordance with the process of the optical palmityl-CoA-deacylase test (25). The following were pipeted into a 1 cm narrow cuvette (cell): TRIS-HCl buffer, pH 7.4, 100 μ Mol, 2,6-dichlorophenol indophenol 30 μg, particle-free tissue extract (105,000 rpm supernatant) corresponding to 4-20 μg protein; by previous dialysis of the tissue extracts (12 hours against 1000 vol. TRIS-HCl 0.005 M, pH 7.4 at 2°C) disturbing "blind" discolorations could be excluded to a great extent. The reaction was started by adding 0.38-0.48 μ Mol acetyl-CoA. End volume:

1.0 ml (preparation of the acetyl-CoA by reaction of CoA-SH with acetic anhydride according to the method of Simon and Shemin (37) and Eggerer (10): a five-fold excess of acetic anhydride is reacted with CoA-SH at pH 7.6-8.0 and room temperature; the reaction ends in a few minutes. After acidifying with 10N HCl to pH 3.0, perforation is done with diethyl ether for 6 hours. The aqueous phase is concentrated to the desired volume and adjusted to pH 4-5). Addition of a little dichlorophenolindophenol (DPI) was sufficient to effect the oxidation of small amounts of free CoAs present in acetyl-CoA. The dependence of DPI-discoloration on the amount of deacylase used is shown in Fig. 3. The specific enzyme activity is given in international milli-units (mμ Mol acetyl-CoA split per min per mg protein). In contrast to earlier reports (44), we found this time an extinction drop (decrease) for DPI $\Delta E_{578} = 25$ for 1 μ Mol cysteine or CoA-SH per ml (pH 7.4). This difference must probably be attributed to the nonuniform quality of different DPI preparations.

By means of the above test, the total acetyl-CoA splitting activity is determined, whereby no more specific statements can be made about the specificity or individuality of the deacylase(s) (17).

Substrate determinations. Acetyl-CoA according to (40,43), taking into account the correction according to (6), CoA by the phosphotransacetylase test according to Stadtman (39), pyruvate (7), lactate (19), acetoacetate (28) and acetaldehyde (3) in the usual manner by optic-enzymatic methods. Acid-insoluble CoA (long-chain acyl-CoA-thioesters) was determined as CoA after alkaline saponification (39).

Products. ATP, CoA, oxalacetic acid, citrate synthase, lactate dehydrogenase, alcohol dehydrogenase, phosphotransacetylase and other bio-

chemicals were supplied by the firm Boehringer & Soehne GmbH, Mannheim. Sulfanilamide was obtained from the firm Fluka, Buchs, Switzerland, and cattle serum albumin from the Behringwerke AG, Marlurg/Lahn. Radioactive compounds were obtained from the Radiochemical Centre, Amersham, England. All other reagents were supplied by E. Merck, Darmstadt.

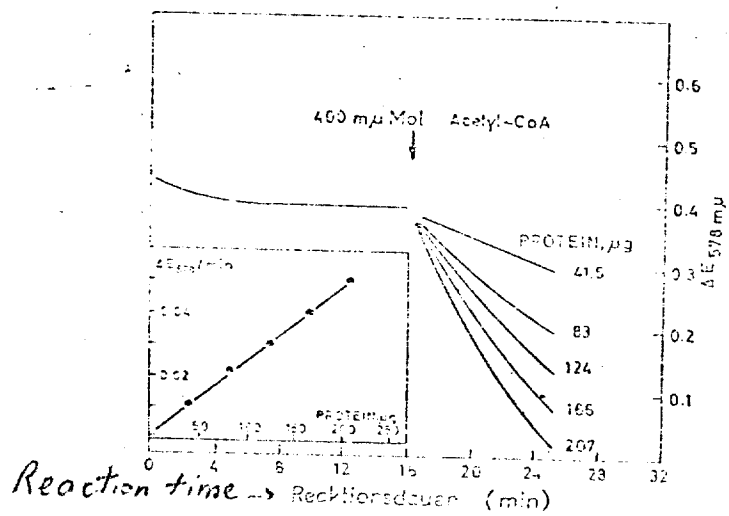


Fig. 3. Optical test for acetyl-CoA-deacylase with dichlorophenolindophenol. Dependence of the discoloration rate (velocity) on the protein concentration (mouse liver extract). Test batch, see method. λ = 578 mu, d = 1 cm, t = 25°C. The enzyme activities are determined by extrapolation of the initial rates (velocities).

Results

Test with intact cells. When fresh ascites tumor cells are shaken in an atmosphere of 95% O_2 and 5% CO_2 in a Krebs-Ringer bicarbonate buffer, the cells liberate (give off) acetate into the medium (Fig. 4). Since no substrate was added to the batch, acetic acid must have been formed from endogenous sources. Acetate formation, which stops after about 100 min, can be substantially increased by addition of pyruvate, as shown in Fig. 5. A quantitative examination of these tests, when taking "idle (blank) formation" into account, shows that exactly one third of the pyruvate added is

converted to acetic acid in 80 min. As Table 1 shows, acetate formation from pyruvate depends on the amount of added pyruvate.

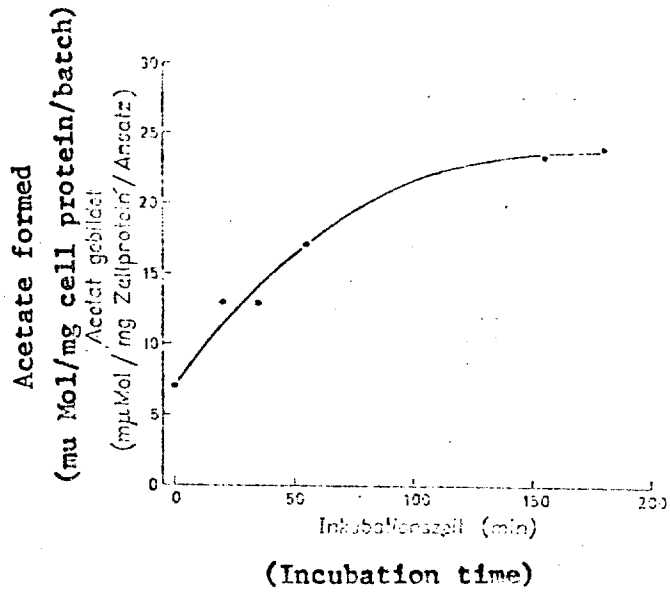


Fig. 4. Acetate formation of ascites tumor cells. Washed tumor cells (MAT-I), corresponding to 70 mg cell protein per batch, were incubated, without addition of substrate under shaking in a Krebs-Ringer-bicarbonate solution under 95% O_2 and 5% CO_2. End volume 3.0 ml, t = 37°C. The batches were stopped at the various time periods shown by addition of perchloric acid (final concentration 3%). Acetic acid was determined in the clearly centrifuged filtrates (see method).

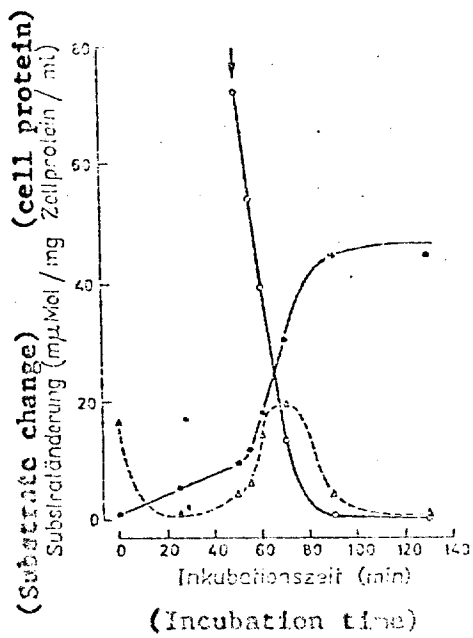


Fig. 5. Pyruvate stimulation of acetate formation by ascites tumor cells. Washed tumor cells (MAT-I), corresponding to 40 mg cell protein per ml incubation medium, were incubated in a Krebs-Ringer bicarbonate solution under a constant gas flow of 95% O_2 and 5% CO_2. For determination of the substrate, aliquot portions of the cell suspension were taken at the various times shown and placed into precooled perchloric acid. After 50 min (arrow), addition of 2.0 u Mol pyruvate per ml. t = 37°C. Acetate •——•; pyruvate ○——○; lactate △- - - △.

Table 1. Dependence of acetate formation on pyruvate concentration. Washed tumor cells (MAT-I) were incubated with increasing amounts of pyruvate as described in the test shown in Fig. 4. Duration of test: 90 min.

① Pyruvatkonzentration	Acetat gebildet (②) mμMol pro mg Zellprotein
$2 \cdot 10^{-3}$ M	40,5
$1 \cdot 10^{-2}$ M	82
$2 \cdot 10^{-2}$ M	106,5

① Pyruvate concentration. ② Acetate formed, in mu Mol/ml per mg cell protein.

In order to obtain more detailed information on the quantitative transformations (reactions) of pyruvate in the tumor cells, the test was repeated with 2-^{14}C-labeled pyruvate (Fig. 6). In this test, 20% of the pyruvate was recovered as acetate and about 45% was recovered as $^{14}CO_2$, which must originate from the end oxidation via the citric acid cycle.

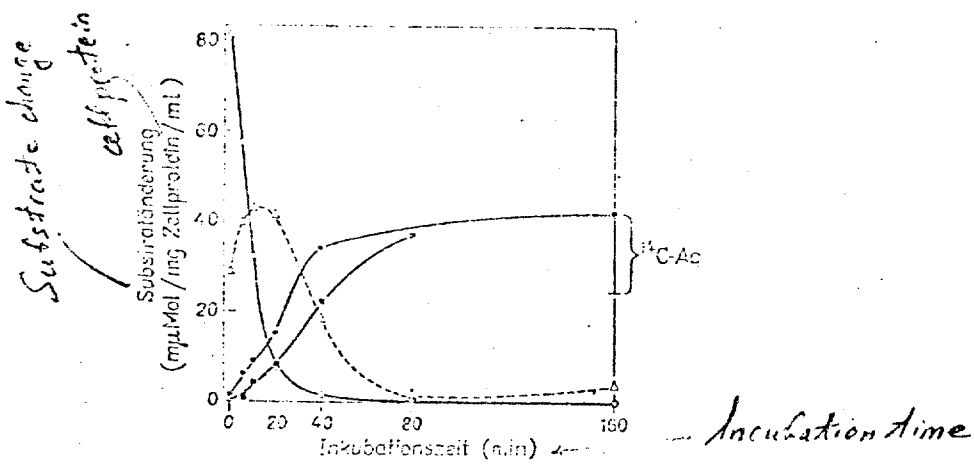


Fig. 6. Breakdown of 2-^{14}C-pyruvate by ascites tumor cells. Washed tumor cells (MAT-I), corresponding to 40 mg cell protein, were incubated in individual batches in a Krebs-Ringer bicarbonate solution under 95% O_2 and 5% CO_2. Stoppage of the reaction with perchloric acid and capture of $^{14}CO_2$ with phenylethylamine was done in the usual manner by injection into the closed reaction vessel. To determine the radioactivity present in the acetate after 160 min, the batch was subjected to microdiffusion. The acetic acid portion formed from pyruvate (^{14}C-Ac) is shown in the figure. Under these test conditions, ca. 1% of the radioactive acetic acid formed goes over into phenylethylamine with $^{14}CO_2$. At the start of the test, addition of 3.26 μ Mol 2-^{14}C-pyruvate per ml. t = 37°C. Further treatment, see test shown in Fig. 4. Acetate ●—●; pyruvate ○—○; lactate △—△; $^{14}CO_2$ □—□.

As can be seen from Figs. 5 and 6, a temporary (transient) accumulation of lactate takes place after addition of pyruvate. Apparently, the tumor cells are not able to oxidize the excess amount of supplied pyruvate sufficiently quickly, so that part of the pyruvate is first converted into lactate (in the manner of an overflow). This phenomenon corresponds to the well-known aerobic glycolysis of ascites tumor cells (41).

When the Ehrlich cells are incubated under nitrogen, no acetate production beyond the range of idle (blank) production takes place under the effect of pyruvate. As Fig. 7 shows, all of the pyruvate is converted into lactate under an aerobic conditions. From this test we can conclude that acetate is not formed by way of a dismutation reaction

$$2 \text{ pyruvate} \longrightarrow 1 \text{ acetate} + \text{lactate} + CO_2,$$

as was observed in other tissues (23).

Mechanism of the aerobic conversion of pyruvate to acetate. According to studies of Holzers *et al.*, a coenzyme A independent conversion of pyruvate to acetate takes place in yeast cells (20). In this reaction, which takes place in the cytoplasm of yeast cells, acetaldehyde is liberated from hydroxyethyl thiamine pyrophosphate and oxidized to acetic acid. The latter, however, is not detected as the end product in yeast, since it is rapidly activated by the active acetate thiokinase.

In order to check whether a similar path of acetate formation might also be considered in ascites cells, we attempted to block the lipoic acid-dependent step hydroxyethyl thiamine pyrophosphate $\longrightarrow$ acetyl-CoA by arsenite (21,32,35), in order to possibly stimulate in this manner the formation of acetic acid. When 1-^{14}C-labeled pyruvate was used, it was found, however, that already decarboxylation is inhibited by arsenite

(Table 2). Thus, it was not possible to further clarify the question of a CoA-independent acetate production from pyruvate by using arsenite.

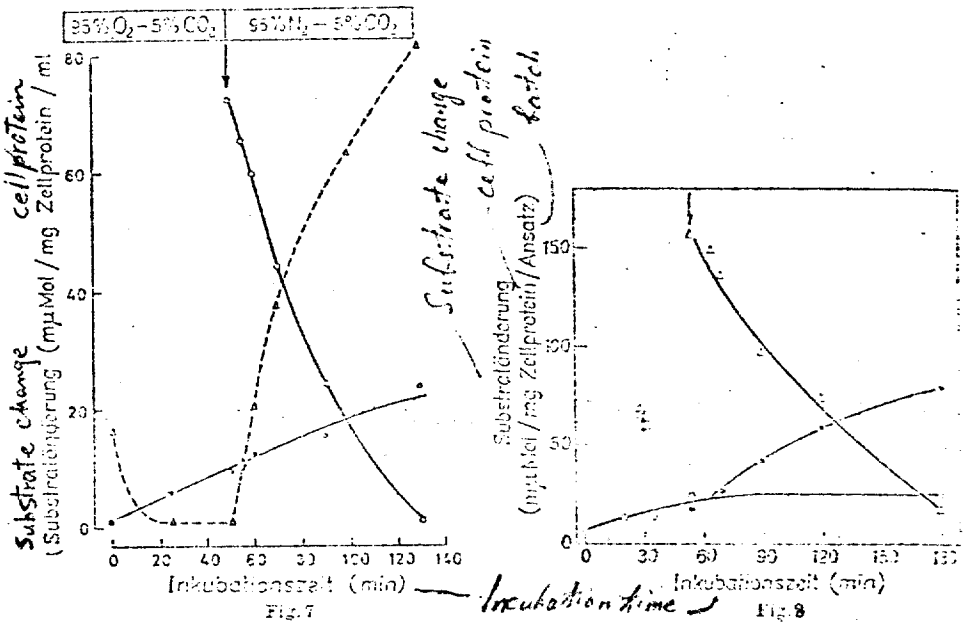


Fig. 7. Absence of a pyruvate-dependent acetate formation under anaerobic conditions. Test conditions, see Fig. 5. After 50 min, change of the gas phase to 95% N_2 and 5% CO_2. Acetate •—•; pyruvate o—o; lactate Δ---Δ.

Fig. 8. Acetate formation by ascites tumor cells from acetaldehyde. Washed tumor cells (MAT-I), corresponding to 70 mg cell protein per batch, were incubated in a Krebs-Ringer bicarbonate solution under 95% O_2 and 5% CO_2. Addition of 4.15 μ Mol acetaldehyde after 55 min (arrow). End volume 3.06 ml. Further treatment as in test shown in Fig. 4. Acetate •—•; acetate without substrate o—o; acetaldehyde Δ—Δ.

Table 2. Effect of arsenite on pyruvate decarboxylation of ascites tumor cells. Incubation of washed tumor cells (MAT-I) in a Krebs-Ringer-phosphate solution under air with 4.32 μ Mol 1-^{14}C-pyruvate. End volume 2.0 ml. t = 37°C. Stoppage of the reaction and capture of the $^{14}CO_2$ formed was done in the usual manner (41). Determination of pyruvate by optic enzymatic means (7).

Arsenite concentration (Mol/1)	$^{14}CO_2$ (in percent of the Imp. used)	Remaining non-volatile ^{14}C (in percent of the Imp. used)	Remaining pyruvate (enzymic) (in percent of the amount used)
0.0	30	65	13
$1.2 \cdot 10^{-4}$	2.72	91.7	82
$2.4 \cdot 10^{-4}$	2.11	93	94
$2.0 \cdot 10^{-3}$	0.47	84	84

We therefore attempted to determine directly the formation of acetaldehyde from pyruvate. During incubation of ascites tumor cells with pyruvate (concentration 0.0033 and 0.01 M) under aerobic conditions, no free acetaldehyde could be detected by optic-enzymatic means. On the other hand, ascites cells are capable of forming acetic acid from acetaldehyde (Fig. 8).

In the arsenite inhibition tests, an increasing inhibition of acetic acid formation from pyruvate was observed, as expected (Fig. 9). At the same time, it was found, however, that tumor cells without added substrate produced more acetate in the presence of arsenite than when incubated without arsenite. This extra amount of acetate must therefore have been formed from endogenous preliminary acetate stages, and probably from the same stages responsible also for the acetate "blank formation" in previous tests (Fig. 4). Assuming that we are dealing with fatty acids in this case, the arsenite effect could be interpreted in such a way that an accumulation of acetyl-CoA takes place as a result of inhibition of the citric acid cycle at the α-ketoglutarate stage, and that acetic acid is formed by enzymatic cleavage of acetyl-CoA. This observation prompted us to conduct further studies aimed at the stimulation of acetic acid formation of tumor cells by long-chain fatty acids.

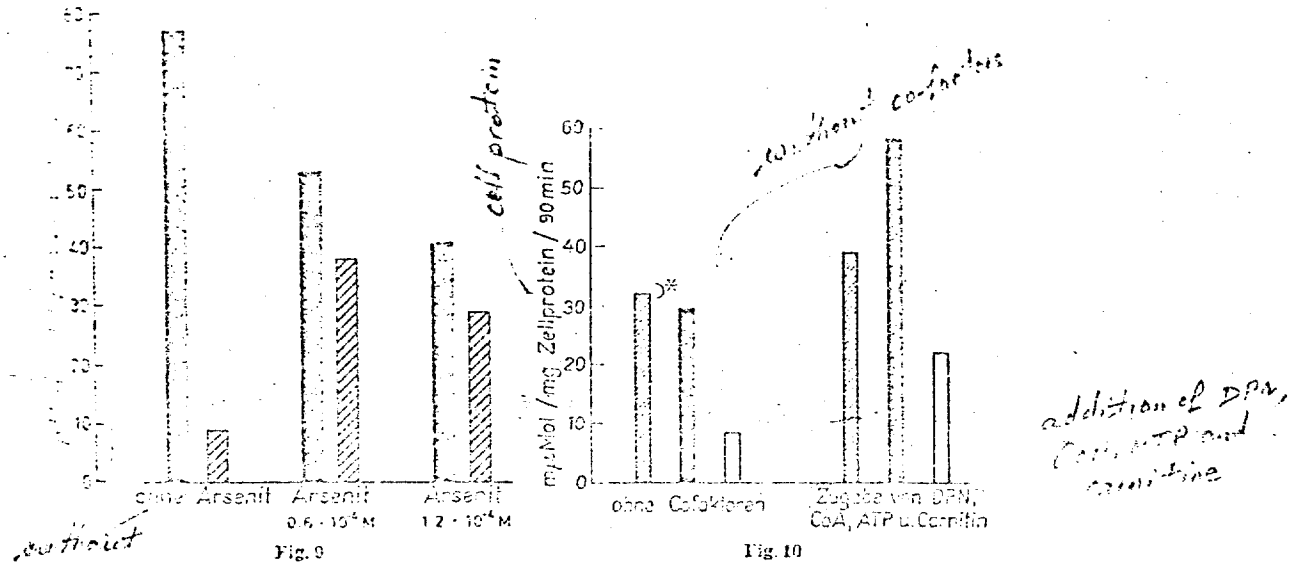


Fig. 9. Acetate formation of ascites tumor cells from pyruvate in the presence of arsenite. Washed tumor cells (MAT-I), corresponding to 55 mg cell protein per batch, were incubated with 0.01 M pyruvate in a Krebs-Ringer-phosphate solution under air. End volume 3.0 ml, t = 37°C. Batches were stopped after 90 min with perchloric acid. ■ -pyruvate 0.01 M; ▨ -no substrate.

Fig. 10. Acetate and CO_2 formation of ascites tumor cells with palmitate as precursor. Effect of cofactors. Washed tumor cells (MAT-I), corresponding to 35 mg cell protein per batch, were incubated for 90 min in a Krebs-Ringer bicarbonate solution under 95% O_2 and 5% CO_2 with 0.001 M 1-^{14}C-palmitate albumin complex (13). ▨ -CO_2; ■-acetate; □-acetate without substrate addition. The CO_2-value designated by an asterisk (*) was obtained from another test, where 0.00033 M palmitate was used.

Acetate formation from fatty acids. From Fig. 10 we can see that acetate formation by ascites cells is also increased, in the same way as by pyruvate, by the addition of long-chain fatty acids. In these tests, 1-^{14}C-palmitate was supplied in the form of an albumin complex, prepared according to (13). From these tests we can also see that optimum fatty acid oxidation takes place only when the cofactors CoA, ATP and DPN are

added. Carnitin provides, in the same way as in other tissues (15, 16), an additional stimulation of both $^{14}CO_2$ and acetic acid formation. It is worth noting that the above cofactors also stimulate acetate production from endogenous sources.

In the same way as in the tests without substrate addition (Fig. 9), acetic acid formation from palmitate by tumor cells is also increased by arsenite. With fluoroacetate (4, 33), on the other hand, we found neither an inhibition of $^{14}CO_2$ formation nor an increase of acetate formation. This can be explained by the fact that fluoroacetate is only sparingly activated in ascites cells, and therefore the required inhibition concentrations of fluoroacetate are apprently not achieved (see also 33, 34).

Comparison of the acetate production of different tumors. The net formation of free acetic acid is not a special property of the mouse ascites tumors used in the investigations described so far. As Fig. 11 shows, other ascites tumor cells can produce varying amounts of acetate, both during incubation without a substrate as well as with pyruvate or palmitate. It is worth noting that acetate formation from pyruvate takes place in thin sections of a solid, transplantable hepatoma (see Note), as shown in Fig. 12. Under identical test conditions, thin sections of normal rat and mouse livers do not liberate any acetic acid. (Note: A transplantable rat liver carcinoma induced by diethylnitrosamine (DENA), which was gragefully supplied to us by Prof. Dr. Druckrey, of the Research Group for Preventive Medicine, Freiburg).

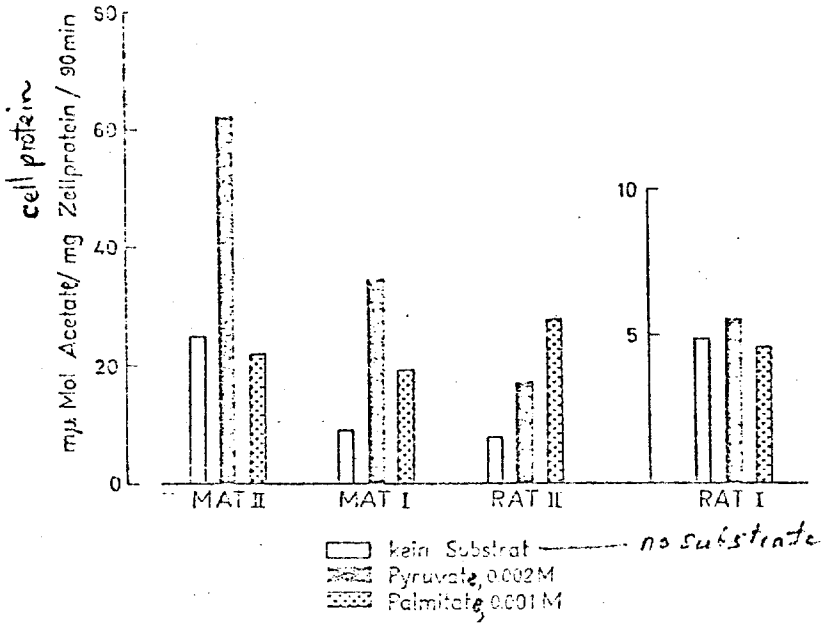


Fig. 11. Acetate formation from pyruvate and palmitate by different tumors. Same conditions as in the tests shown in Fig. 9 and 10.

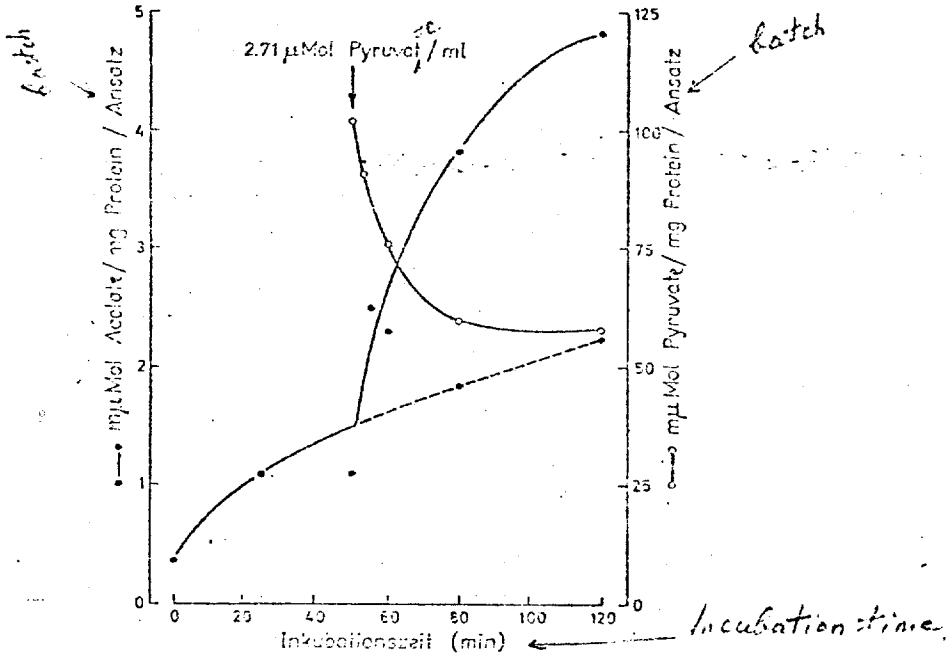


Fig. 12. Stimulation of acetate formation by pyruvate in thin sections of an hepatocarcinoma. Thin sections of hepatoma (see Note above), corresponding to 300 mg moist (wet) weight, were incubated in a Krebs-Ringer bicarbonate solution in single batches of 3.0 ml end volume under 95% O_2 and 5% CO_2. Addition of 2.71 μ Mol pyruvate per ml after 50 min (arrow), t = 37°C. Further treatment of samples similar to test shown in Fig. 4. The broken-line curve shows acetate formation in the absence of pyruvate.

Content of coenzyme A derivatives. We were unable to determine acetyl-CoA by the Ochoa test (40, 43) in $HClO_4$ extracts from lyophilizates of MAT-I. The content of free-CoA amounted to 40 mμ Mol per gram dry weight, as compared to 27.8 mu Mol of acid-insoluble CoA per gram dry weight. The same tumor passage contained 6.16 μ Mol acetate per gram dry weight.

Tests with tumor extracts. Following the results obtained with intact cells, the most plausible explanation of why the net formation of acetate takes place is an imbalance (disproportion) between acetate activation and acetyl-CoA splitting. An experimental basis for this concept (assumption) was provided by determinations of the activity of the enzymes acetate thiokinase and acetyl-CoA-deacylase. In fact, as can be seen from Table 3, the activity of thiokinase in tumor cells is extremely low and is equal to only about 1/60th of the activity of the liver. The deacylase/thiokinase ratio in the liver is about 1.4, whereas in the MAT-I tumor this ratio is 64. As Table 3 shows, a similarly low thiokinase activity is found not only in ascites tumor cells, but also in the rat hepatoma examined by us. In order to check whether, along with these quantitative differences, any qualitative changes of the above enzymes are present, we determined their affinity towards acetate and acetyl-CoA. According to the substrate saturation curves (Fig. 13 and 14), however, no significnat differences could be found. Thus, the Michaelis constant of liver thiokinase lies at 1.8×10^{-4} M acetate, and that of thiokinase from ascites tumor at 1.6×10^{-4} M acetate. For liver deacylase $K_m = 0.6 \times 10^{-4}$ M acetyl-CoA, and for tumor deacylase $K_m = 0.3 \times 10^{-4}$ M acetyl-CoA.

Table 3 Specific activities and Michaelis constants of acetyl thiokinase and acetyl-CoA-deacylase in liver and tumor extracts.

		Thiokinase[1]	Deacylase[2]	Deacylase / Thiokinase
Mouse liver	Specific activity (sp. act.)	6.7	9.6	1.43
	K_m	1.8×10^{-4} M (Acetate)	0.6×10^{-4} M (Acetyl-CoA)	
Ehrlich-Ascites-Carcinoma	Specific activity (sp. act.)	0.11	7.05	64
	K_m	1.6×10^{-4} M (Acetate)	0.3×10^{-4} M (Acetyl-CoA)	
DENA-liver carcinoma	Specific activity (sp. act.)	0.36	3.5	9.7
	K_m			

[1] Enzyme determination at 37°C. --- [2] Enzyme determination at 25°C.

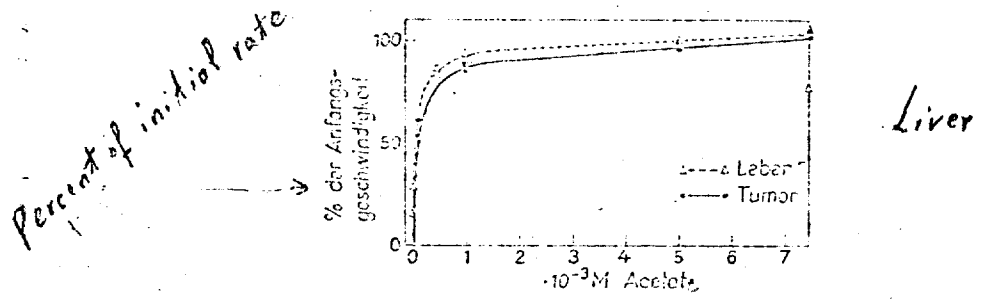


Fig. 13. Substrate saturation curve of acetate thiokinase. Comparison of liver and tumor. Test procedure, see method. Mouse liver Δ---Δ ; MAT-I •——• .

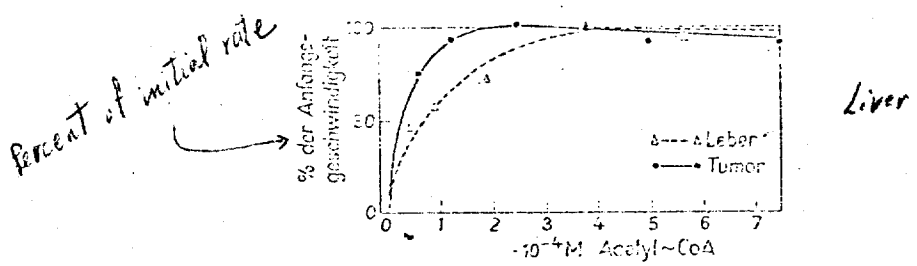


Fig. 14. Substrate saturation curve of acetyl-CoA-deacylase. Comparison of liver and tumor. Test procedure, see method. Mouse liver Δ---Δ; MAT-I •——• .

Discussion

The first question arising from the studies described here is whether the aerobic production of free acetic acid by ascites tumor cells (as well as the high aerobic glycolysis) can be considered as a general characteristic of malignant tumors, or whether it represents only a special case of definite tumors. In the present study, this feature was observed in four different ascites tumors, including two carcinomas, one sarcoma, one hepatoma, and one solid hepatoma. While our studies were in progress, we took notice with special interest of a communication by F. Lynens, who had noted, already in 1940, the formation of acetate in sections of the rat Jensen sarcoma (26). At the same time, Elliot et al. (12) stated the assumption that acetic acid is formed in the oxidation of pyruvate by various tumors (Philadelphia No. 1 sarcoma, Walker 256 carcinoma), although they were unable to provide an experimental proof of this fact. Freedman and Graff (14) also came to the conclusion, on the basis of their isotope distribution studies, that a C-2 product is formed from pyruvate in thin sections of a Murphy-Sturm lymphosarcoma.

Our measurements of the enzymes acetate thiokinase and acetyl-CoA-deacylase appear to give a plausible answer to the question as to how the net production of acetate takes place in tumor cells. It is conceivable that thiokinase and deacylase in normal tissues are synchronized in such a way, with respect to each other, that no liberation of acetic acid takes place (Fig. 15). In a tumor, however, the activity of thiokinase is particularly weak (low) in relation to deacylase (compare Table 3), so that acetate activation is no longer in step (or can no longer keep up) with acetyl-CoA splitting. The result is the net formation of free acetic acid.

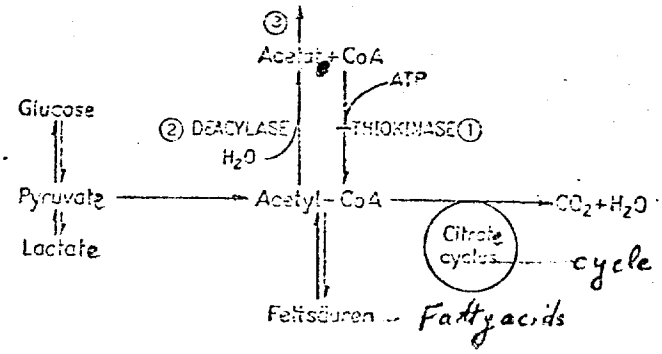


Fig. 15. Mechanism of acetate formation in tumors. In view of the low thiokinase activity of tumor cells 1, acetate activation can no longer keep up with acetyl-CoA splitting 2, thus leading to an interruption (break) of the acetate cycle and the net formation of free acetic acid 3.

The low supply of acetate-activating enzyme found in the tumors studied by us also provides an explanation for numerous earlier findings pointing to the slight (poor) utilization of acetate by a whole series of tumors. Thus, it was found already in 1935 that acetate is only poorly oxidized by sections of Philadelphia No. 1 sarcoma and rat Walker 256 carcinoma (11). On the basis of tests with 1-^{14}C-acetate on sections of Flexner-Jobling and Walker 256 carcinoma, Pardee et al. (31) came to the conclusion that activation of acetic acid, measured on $^{14}CO_2$, is only weak. This fact was then confirmed by Busch (8,9) during in vivo studies on tumor-bearing rats (Jensen, Walker 256, Flexner-Jobling carcinoma, lymphoma). After injection of 1-^{14}C-acetate, the bulk of the radioactivity remained present in the tumors as acetic acid, whereas in other tissues 70-100% of the activity was transferred into non-volatile products during the same time. With and without a malonate pretreatment, a very low $^{14}CO_2$ formation from acetate was noted, which led the authors to assume that practically no acetic acid is utilized by tumors. Observations made by Medes et al. (27), showing that thin sections of various tumors can incorporate very little acetate into

lipids, also point in the same direction.

The concept that part of the acetyl-CoA is constantly hydrolyzed and thus removed from the citric acid cycle allows us to entertain some causal thoughts in regard to the well-known high aerobic glycolysis of malignant cells [(41), for a review see (1)]. Indeed, the continuous attrition of acetyl-CoA would lead to a deficiency in respiratory energy, a condition which could induce, by compensatory means, the start of aerobic glycolysis. In this case, the primary disturbance would thus consist in a limited enzyme defect, namely in a depletion of acetate thiokinase in tumor cells. In order to further clarify this question, it would appear necessary to extend studies to include the maximum possible number of different tumors, and primary tumors showing the slightest (minimum) deviations, such as, for example, Morris hepatoma 5123 (29), and also other tumors.

Siperstein and Fagan (36) have reported some data on acetate metabolism in hepatomas. These data show that formation of $^{14}CO_2$ is greatly reduced, as compared to normal liver, whereas acetate incorporation into fatty acids and the cholesterol content in tumors is even increased. These findings would not indicate a disruption of acetate activation, although it appears that more precise enzymatic studies are required.

We wish to express our gratitude for the generous support of these studies to the German Research Association in Bad Godesberg, the Bavarian State Ministry for Education and Culture, and to the Farbwerke Hoechst, Frankfurt-Hoechst.

Bibliography

[1] AISENBERG, A. C.: The Glycolysis and Respiration of Tumors. New York and London: Academic Press 1961.

[2] BEISENHERZ, G., H. J. BOLTZE, TH. BÜCHER, R. CZOCK, K. H. GARBADE, E. MEYER-ARENDT u. G. PFLEIDERER: Z. Naturforsch. 8b, 555 (1953).

[3] BERGMEYER, H. U.: In: Methoden der enzymatischen Analyse, S. 290. Weinheim: Verlag Chemie 1962.

[4] BRADY, R. O.: J. biol. Chem. 217, 213 (1955).

[5] BRAY, G. A.: Analyt. Biochem. 1, 279 (1960).

[6] BUCKEL, W., u. H. EGGERER: Biochem. Z. 343, 29 (1965).

[7] BÜCHER, TH., R. CZOCK, W. LAMPRECHT u. E. LATZKO: In: Methoden der enzymatischen Analyse, S. 253. Weinheim: Verlag Chemie 1962.

[8] BUSCH, H.: Cancer Res. 13, 789 (1953).

[9] —, and H. A. BALTRUSH: Cancer Res. 14, 448 (1954).

[10] EGGERER, H.: Pers. Mitteilung.

[11] ELLIOTT, K. A. C., M. P. BENOY, and Z. BAKER: Biochem. J. 29, 1937 (1935).

[12] —, and M. E. GREIG: Biochem. J. 31, 1921 (1937).

[13] FILLERUP, D. L., I. C. MOLLIORE, and J. F. MEAD: J. biol. Chem. 233, 98 (1958).

[14] FREEDMAN, A. D., and S. GRAFF: J. biol. Chem. 233, 292 (1958).

[15] FRITZ, I. B.: Physiol. Rev. 41, 52 (1961).

[16] —, and K. B. N. YUE: J. Lipid Res. 4, 279 (1963).

[17] GERGELY, J., P. HELE, and C. V. RAMAKRISHNAN: J. biol. Chem. 198, 323 (1952).

[18] HOCHHEUSER, W., H. WEISS u. O. WIELAND: Z. klin. Chem. 2, 175 (1964).

[19] HOHORST, H. J.: In: Methoden der enzym. Analyse, S. 266. Weinheim: Verlag Chemie 1962.

[20] HOLZER, H., u. H. W. GOHDE: Biochem. Z. 329, 175 (1957).

[21] — — Biochem. Z. 329, 192 (1957).

[22] JONES, M. E., S. BLACK, R. M. FRYNN, and F. LIPMANN: Biochim. biophys. Acta (Amst.) 12, 141 (1953).

[23] KEBES, H. A., and W. A. JOHNSON: Biochem. J. 31, 645 (1937).

[24] LUNDQUIST, F., U. FUHMANN, E. KLÄNING, and H. RASMUSSEN: Biochem. J. 72, 409 (1959).

[25] LYNEN, F., P. A. SBERE, and W. SEUBERT: Biochim. biophys. Acta (Amst.) 33, 313 (1959).

[26] — Unveröffentlicht.

[27] MEDES, G., A. J. THOMAS, and S. WEINHOUSE: Cancer Res. 13, 27 (1953).

[28] MELLANBY, J., u. D. H. WILLIAMSON: In: Methoden der enzymatischen Analyse, S. 454. Weinheim: Verlag Chemie 1962.

[29] MORRIS, H. P., SZBANSKY, H., B. P. WAGNER, and H. M. DYER: Cancer Res. 20, 1252 (1960).

[30] NUMA, S., M. MATSUHASHI u. F. LYNEN: Biochem. Z. 334, 253 (1961).

[31] PARDEE, A. B., C. HEIDELBERGER, and V. R. POTTER: J. biol. Chem. 186, 625 (1950).

[32] PETERS, R. A., H. M. SINCLAIR, and R. H. S. THOMPSON: Biochem. J. 40, 516 (1946).

[33] POTTER, V. R., and H. BUSCH: Cancer Res. 10, 353 (1950).

[34] REISS, O. K., L. HELLERMANN, J. A. AMBROSE, C. I. SMITH, M. K. GEY, and A. E. IBER: J. biol. Chem. 237, 992 (1962).

[35] SCHEUBER, G., G. KOHLHAW, H. W. GOHDE u. H. HOLZER: Biochem. Z. 339, 83 (1963).

[36] SIDERSTEIN, M. D., and V. M. FAGAN: Cancer Res. 24, 1198 (1964).

[37] SOBON, E. J., and D. SHEMIN: J. Amer. chem. Soc. 75, 2520 (1953).

[38] SOODAK, M., and F. LIPMANN: Fed. Proc. 7, 199 (1948).

[39] STADTMAN, E. R.: Aus: MICHAL, G., u. H. U. BERGMEYER. In: Methoden der enzymatischen Analyse, S. 517. Weinheim: Verlag Chemie 1962.

[40] STERN, I. R., B. SHAPIRO, E. R. STADTMAN, and S. OCHOA: J. biol. Chem. 193, 703 (1951).

[41] WARBURG, O.: Über den Stoffwechsel der Tumoren. Berlin: Springer 1926.

[42] WIELAND, O., u. I. NEUFELDT: Hoppe-Seylers Z. physiol. Chem. 324, 101 (1961).

[43] —, u. L. WEISS: Biochem. biophys. Res. Commun. 10, 333 (1963).

[44] — L. WEISS u. I. EGER-NEUFELDT: Biochem. Z. 339, 501 (1964).

[45] WERBA, H., u. H. RABES: Z. Krebsforsch. 65, 316 (1963).

[46] ZAJDELA, F.: Colloque Franco-Soviétique, Moscou, Juillet 1962. p. 49. Paris: Gauthier-Villars 1963.

Received for publication
on 13 October 1965.

Biochemische Zeitschrift 344, 87—102 (1966)

Essigsäure als Endprodukt des aeroben Krebsstoffwechsels

DIETER HEPP, EVA PRÜSSE, HANNS WEISS* und OTTO WIELAND**

Laboratorium für Klinische Chemie
und Biochemie der II. Medizinischen Universitätsklinik, München

Eingegangen am 13. Oktober 1965

In four different strains of ascites tumors and in one solid hepatocarcinoma acetate was observed to constitute an end-product of aerobic metabolism of pyruvate and palmitate. According to enzymatic studies with soluble tumor extracts there is a striking decrease in the activity of acetate thiokinase as compared to liver tissue which does not form acetate. On the other hand, the activity of the acetyl-CoA splitting enzyme(s) is undiminished in tumor extracts. It is assumed that net formation of acetate in tumors is the result of an imbalance of the acetate activating enzyme and the acetyl-CoA splitting enzyme(s). The implications of the permanent loss of respiratory energy caused by this defect in the tumor enzyme pattern with regard to the development of aerobic glycolysis of malignant tissues is discussed.

Bei vier verschiedenen Ascitestumoren und bei einem soliden Lebercarcinom wurde das Auftreten von Essigsäure als eines der Endprodukte der Pyruvat- und Fettsäureoxydation beobachtet. Enzymstudien mit Tumorextrakten ergaben auffallend geringe Aktivitäten der Acetat-thiokinase im Vergleich zu nicht acetatproduzierenden Geweben, wie z. B. Leber, während die enzymatische Spaltung von Acetyl-CoA durch Deacylase(n) im Tumor unverändert war. Die Nettobildung von Acetat dürfte das Ergebnis eines Mißverhältnisses von Acetyl-CoA-Spaltung und Acetatakti-vierung darstellen. Mögliche Zusammenhänge dieser Anomalie, die einen kontinuierlichen Verschleiß an Atmungsenergie bedeutet, mit der Glykolysesteigerung der Tumoren werden diskutiert.

Bei Untersuchungen über den Essigsäurestoffwechsel tierischer Gewebe [18] war aufgefallen, daß Mäuseascites-Tumorzellen in vitro auffallend hohe Mengen an Acetat produzieren. Wir sind dieser Erscheinung weiter nachgegangen mit dem Ziel, die der vermehrten Acetatbildung der Tumorzellen zugrundeliegenden Mechanismen aufzuklären und damit zu einem Urteil zu kommen, ob es sich hier um eine Anomalie des Tumorstoffwechsels von breiterem Interesse handeln könnte. Im folgenden berichten wir über die Ergebnisse dieser Studien.

* Die Arbeit enthält Auszüge der Dissertation von H. WEISS, München (1964/65).

** Auszugsweise vorgetragen auf dem „Fourth International Symposium on Regulation of Enzyme Activity and Synthesis in Normal and Neoplastic Tissues" Indianapolis, 4.—5. X. 1965 und der Herbsttagung der Deutschen Gesellschaft für Physiologische Chemie, Berlin, 6.—9. X. 1965.

Methodik

Tumoren. Die untersuchten Ascites-Tumoren werden im folgenden unter der Bezeichnung geführt[1]:

a) MAT I: Ehrlich-Ascites-Tumor der Maus, diploid.

b) MAT II: Ehrlich-Ascitestumor der Maus, tetraploid. Die Stämme wurden am Pathologischen Institut der Universität München gezüchtet [45].

c) RAT I: Yoshida-Ascites-Sarkom, Ratte, Stamm RY 144 [45].

d) RAT II: Zajdela-Ascites-Hepatom, Ratte [46].

MAT I, der am intensivsten studierte Tumor, wurde in 34 Passagen auf NMRI-Mäusen (Lieferant W. Gassner, München-Ottobrunn) weitergezüchtet und zeigte von Passage zu Passage keine nennenswerten Unterschiede in Zellzahl und Zellvolumen.

Gewinnung der Tumorzellen. Der Ascites wurde am 7.—11. Tag nach Transplantation in üblicher Weise durch Punktion der Bauchhöhle durch das freipräparierte Peritoneum mit einer Rekordspritze entnommen und sofort dreimal mit eiskalter, steriler Ringer-Lösung an der Zentrifuge bei 4° C und etwa 1000 g gewaschen. Sanguinolente Proben wurden verworfen. Im Falle des RAT II ließen sich Erythrocyten durch kurzes niedertouriges Zentrifugieren weitgehend entfernen.

Für Inkubationsversuche intakter Zellen wurden die geernteten Tumorzellen unmittelbar im Anschluß an die dritte Waschung herangezogen. Zur Gewinnung von Proteinextrakten wurden die gewaschenen, gepackten Zellen zum Teil gefriergetrocknet oder bei — 15° C in der Tiefkühltruhe aufbewahrt.

Versuche mit intakten Tumorzellen. Die Inkubation der frischen Zellen führten wir im allgemeinen in kleinen Erlenmeyer-Kölbchen (Volumen 25 ml) oder in Warburg-Gefäßen durch. Für die Isotopenexperimente verwendeten wir die an anderer Stelle beschriebenen Gefäße [42] dahingehend geändert, daß der Alkalienthaltende Glaseinsatz herausnehmbar gestaltet wurde, so daß er samt Inhalt in das Meßgefäß mit der Scintillationsflüssigkeit versenkt werden konnte. Die Gewebemengen wurden entweder durch Einwaage oder durch Pipettieren der gleichmäßig verteilten Zellsuspensionen (Magnetrührer) abgemessen. Alle Inkubationsversuche liefen unter Schütteln im thermokonstanten Wasserbad bei 37° C.

Die gepackten Zellen wurden zur Proteinbestimmung nach der Biuret-Methode [2] in einen Glashomogenisator eingewogen und in einem definierten Volumen homogenisiert. Ein Aliquot dieses Gesamthomogenats wurde eingesetzt.

Im Falle der Leber- und Tumordünnschnitte wurde ebenso verfahren.

Versuche mit Tumorextrakten. Zur Bereitung eiweißhaltiger Rohextrakte für die Enzymuntersuchungen wurden die gewaschenen Tumorzellen erst in flüssiger Luft durchgefroren und im Potter-Glashomogenisator oder in einem Messerhomogenisator[2] mit der jeweiligen Pufferlösung im Verhältnis 1 : 3 scharf aufgeschlossen. Die Extraktion der Lyophilisate erfolgte auf gleiche Weise. (Verhältnis Puffer zu Trockengewicht = 25 : 1 Vol/Gew.) Die Rohextrakte wurden in der Spinco-Zentrifuge bei 105000 g abzentrifugiert. Der Proteingehalt der zellfreien Extrakte wurde mit der Biuret-Methode [2] bestimmt.

Acetatbestimmung. Die Essigsäure bestimmten wir nach dem von LUNDQUIST angegebenen Verfahren [24]. Hierbei wird die Essigsäure zunächst durch Conway-Mikrodiffusion aus den enteiweißten Proben abgetrennt und anschließend im

[1] Die Tumoren a), b) und c) wurden uns freundlicherweise von Herrn Professor H. WERBA, Pathol. Institut der Universität München, Tumor d) von Herrn Professor G. RUHENSTROTH-BAUER, Max Planck-Institut für Biochemie, München, zur Verfügung gestellt.

[2] Fa. MSE Ltd., London, W. W. I., England.

Sulfanilamid-Acetylierungstest nach SOODAK u. LIPMANN [38] enzymatisch bestimmt. Die mit 1 Vol 6% Perchlorsäure gestoppten und zentrifugierten Inkubationsansätze wurden nach der Originalvorschrift [24] mit 6 N NaOH und 0,5 M Na-Citratpuffer, pH 3,0 auf pH 3 eingestellt (Eisbad!) und von dieser Lösung je 1 ml für die Mikrodiffusion eingesetzt. Bezüglich weiterer Details sei auf die Originalvorschrift [24] und auf eine frühere Veröffentlichung [18] verwiesen. Jede Bestimmungsreihe wurde durch eine 6-Punkteichkurve mit Standard-Acetatlösung kontrolliert.

Enzymbestimmungen. a) Acetat-thiokinase. Für die Bestimmung der Acetat-thiokinase in den Tumorextrakten erwies sich die Hydroxamsäuremethode [22] als zu unempfindlich. Es wurde deshalb eine Isotopenmethode ausgearbeitet, die auf folgenden Reaktionen beruht:

$$1\text{-}^{14}\text{C-Acetat} + \text{ATP} + \text{CoA} \xrightarrow{\text{Ac-thiokinase}} 1\text{-}^{14}\text{C-Acetyl-CoA} + \text{AMP} + \text{PP} \quad (1)$$

$$1\text{-}^{14}\text{C-Acetyl-CoA} + \text{Oxalacetat} + \text{H}_2\text{O} \xrightarrow{\text{Citratsynthase}} 1\text{-}^{14}\text{C-Citrat} + \text{CoA} \quad (2)$$

Das in Reaktion (Gl. 1) durch die enzymatische Aktivierung von radioaktiv markiertem Acetat gebildete 1-^{14}C-Acetyl-CoA wird in der Hilfsreaktion (Gl. 2) mit Citratsynthase und Oxalacetat in markiertes Citrat umgewandelt. Letzteres kann

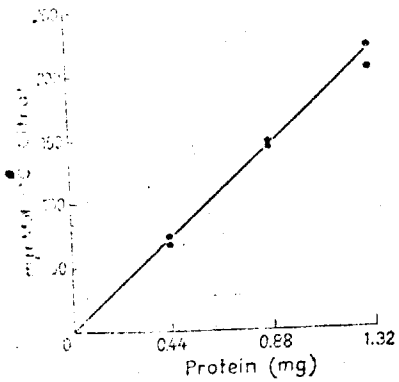


Fig. 1

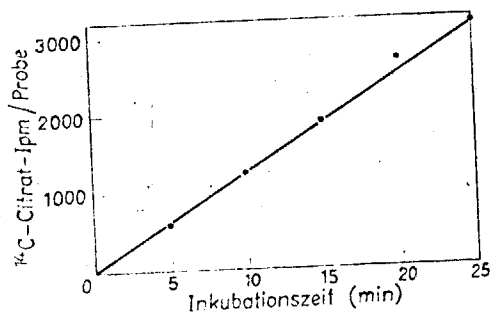


Fig. 2

Fig. 1. *Isotopentest zur Bestimmung der Acetat-thiokinase.* Eichkurve mit steigenden Mengen eines partikelfreien Extraktes aus Mäuseleber. Zusammensetzung des Tests vgl. Methodik

Fig. 2. *Isotopentest zur Bestimmung der Acetat-thiokinase.* Abhängigkeit der ^{14}C-Citratbildung von der Inkubationsdauer. Eingesetzte Proteinmenge (partikelfreier Tumorzellextrakt —MAT I) 4,1 mg pro Ansatz. Weitere Zusammensetzung des Tests vgl. Methodik

durch Fällung als Silbersalz quantitativ aus dem Reaktionsansatz isoliert und gemessen werden. Der Acetateinbau ist bei diesem Vorgehen der eingesetzten Menge an Acetat-thiokinase innerhalb eines weiten Bereiches proportional (Fig. 1). Die Zeitabhängigkeit des Testes zeigt Fig. 2.

Testansatz. In Zentrifugengläser 13×98 mm wurden hintereinander einpipettiert: TRIS-HNO_3-Puffer, pH 7,4 60 µMol, Glutathion-SH 10 µMol, ATP 5 µMol, $MgSO_4$ 5 µMol, CoA-SH 0,4 µMol, Oxalacetat 18 µMol, 1-^{14}C-Acetat 5 µMol (entsprechend ca. 960000 Ipm.), krist. Citratsynthase 20 µg, Rohextrakt (entspricht partikelfreier 105000 g-Überstand) entsprechend 0,4—6 mg Protein. Endvolumen 1 ml, Inkubation 20 min bei 37° C. Die Ansätze werden durch Zugabe von

0,2 ml 0,5 M Citronensäure und 5 min Kochen im Wasserbad gestoppt, nach Abkühlen mit 2 N KOH auf pH 5 eingestellt (Indicatorpapier) und mit 2 ml $AgNO_3$-Reagens versetzt (5 g $AgNO_3$ in ca. 80 ml Wasser lösen, 4 ml 0,5 M TRIS-Lösung und 0,15 ml 2 N HNO_3 zugeben und mit Wasser ad 100 ml auffüllen). Nach Zusatz von 4 ml Aceton-Alkohol 1:1 und 10 min Stehen im Dunkeln ist die Fällung beendet. Den Niederschlag wäscht man dreimal an der Zentrifuge mit einer Mischung von Aceton-Alkohol-Essigsäure (4 M) im Verhältnis 10:10:1 und löst ihn in 1 ml 0,1 N HNO_3. Nach Verdünnen mit 1 ml H_2O entfernt man den Silberüberschuß durch Fällung mit 0,1 ml 5 N HCl und Zentrifugieren. Vom Überstand wurden 0,5 ml in ein Schraubgläschen für den Packard-Scintillationszähler einpipettiert und nach Zusatz von 0,2 ml 4 M Essigsäure bei 80° C zur Trockne eingedampft. Zur Zählung wurde der Rückstand erst mit 0,1 ml 0,1 N NaOH gelöst und dann mit 10 ml Brayscher Lösung [5] aufgefüllt. Als Blindwert diente ein zum Zeitpunkt 0 gestoppter und in der beschriebenen Weise weiter behandelter Ansatz. Er enthielt ca. 0,2 pro Mille der eingesetzten Radioaktivität.

Bei den Enzymbestimmungen lagen die Zählausbeuten in gleicher Höhe wie mit wäßrigen ^{14}C-Acetat-Standardlösungen, so daß auf eine Korrektur verzichtet werden konnte[3]. Die Acetat-thiokinaseaktivität ist angegeben in internationalen milli-Einheiten (mµMol Acetateinbau pro min pro mg Protein). Beim Vergleich des Isotopentestes mit der Hydroxamsäuremethode [22, 30], der mit Leberextrakten durchgeführt wurde, ergaben sich übereinstimmende Werte.

b) Acetyl-CoA-deacylase. Für die Bestimmung der Acetyl-CoA-deacylase wurde in Anlehnung an das Verfahren des optischen Palmityl-CoA-deacylasetestes [25] die Entfärbung von Dichlorphenolindophenol durch das bei der Spaltung freiwerdende CoA-SH gemessen. In eine 1 cm-Schmal-Küvette wurden pipettiert: TRIS-HCl-Puffer, pH 7,4 100 µMol, 2,6-Dichlorphenol-indophenol 30 µg, partikelfreier Gewebeextrakt (105000 g-Überstand) entsprechend 4—20 µg Protein; durch vorausgehende Dialyse der Gewebsextrakte (12 Std gegen 1000 Vol TRIS-HCl 0,005 M pH 7,4 bei 2° C) konnten störende „Blind"-Entfärbungen weitgehend ausgeschaltet werden. Start der Reaktion durch Zusatz von 0,38—0,48 µMol Acetyl-CoA. Endvolumen: 1,0 ml. (Herstellung des Acetyl-CoA durch Umsetzung von CoA-SH und Essigsäureanhydrid in Anlehnung an die Methode von SIMON u. SHEMIN [37] nach ECCERER [10]: In ca. fünffachem Überschuß wird Essigsäureanhydrid mit CoA-SH bei pH 7,6—8,0 und Zimmertemperatur umgesetzt; nach wenigen Minuten ist die Reaktion beendet. Nach Ansäuern mit 10 N HCl auf pH 3,0 wird 6 Std mit Diäthyläther perforiert. Die wäßrige Phase wird auf das gewünschte Volumen konzentriert und auf pH 4—5 eingestellt.) Zur Oxydation kleiner im Acetyl-CoA vorhandener Mengen freien CoAs genügte der Zusatz von wenig Dichlorphenolindophenol. Die Abhängigkeit der DPI-Entfärbung von der eingesetzten Deacylasemenge zeigt Fig. 3. Die spez. Enzymaktivität ist in internationalen milli-Einheiten (mµMol Acetyl-CoA gespalten pro min pro mg Protein) angegeben. Entgegen früheren Angaben [44] fanden wir diesmal eine Extinktionsabnahme für DPI $\Delta E_{578} = 25$ für 1 µMol Cystein oder CoA-SH pro ml (pH 7,4). Dieser Unterschied ist vermutlich auf die uneinheitliche Qualität verschiedener DPI-Präparate zurückzuführen.

Nach dem vorliegenden Test wird die gesamte Acetyl-CoA-spaltende Aktivität bestimmt, wobei keine näheren Aussagen über Spezifität oder Individualität der Deacylase(n) [17] gemacht werden können.

Substratbestimmungen. Acetyl-CoA nach [40, 43] unter Berücksichtigung der Korrektur nach [6], CoA im Phosphotransacetylasetest nach STADTMAN [39], Pyru-

[3] Alle Radioaktivitätsmessungen wurden im Tri-Carb-Scintillationszähler, Modell 3003 der Fa. Packard, La Grange, Illinois, durchgeführt.

vat [7], Lactat [19], Acetacetat [28] und Acetaldehyd [3] in üblicher Weise optisch-enzymatisch. Säureunlösliches CoA (langkettige Acyl-CoA-thioester) nach alkalischer Verseifung als CoA [39].

Präparate. ATP, CoA, Oxalessigsäure, Citratsynthase, Lactatdehydrogenase, Alkoholdehydrogenase, Phosphotransacetylase und sonstige Biochemikalien waren

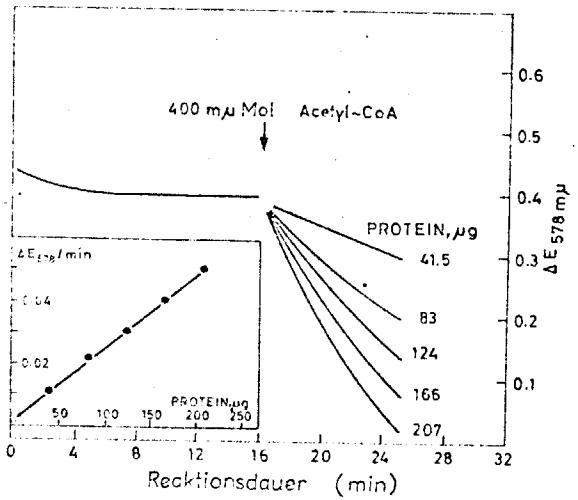


Fig. 3. *Optischer Test auf Acetyl-CoA-deacylase mit Dichlorphenolindophenol.* Abhängigkeit der Entfärbungsgeschwindigkeit von der Proteinkonzentration (Mäuseleberextrakt). Testansatz vgl. Methodik. $\lambda = 578$ mμ, $d = 1$ cm, $t = 25°$ C. Die Enzymaktivitäten werden durch Extrapolation der Anfangsgeschwindigkeiten ermittelt

Produkte der Fa. Boehringer & Soehne GmbH, Mannheim. Sulfanilamid bezogen wir von der Fa. Fluka, Buchs, Schweiz, Rinderserumalbumin von den Behringwerken AG, Marburg/Lahn. Die radioaktiven Verbindungen stammten von The Radiochemical Centre, Amersham, England. Alle übrigen Reagentien wurden von E. Merck, Darmstadt, bezogen.

Ergebnisse

Versuche mit intakten Zellen. Schüttelt man frische Ascites-Tumorzellen in einer Atmosphäre von 95% O_2 : 5% CO_2 in Krebs-Ringer-Bicarbonatpuffer, so geben die Zellen Acetat an das Medium ab (Fig. 4). Da dem Ansatz kein Substrat zugesetzt wurde, muß die Essigsäure aus endogenen Quellen entstanden sein. Die Acetatbildung, die nach etwa 100 min zum Stillstand kommt, läßt sich durch Zugabe von Pyruvat beträchtlich steigern, wie Fig. 5 zeigt. Die quantitative Betrachtung dieser Versuche unter Berücksichtigung der „Leerbildung" ergibt, daß rund ein Drittel der zugesetzten Pyruvatmenge in 80 min in Essigsäure umgewandelt wurde. Wie Tab. 1 zeigt, ist die Acetatbildung aus Pyruvat von der Menge an zugesetztem Pyruvat abhängig.

Um näheren Aufschluß über die quantitativen Umsetzungen des Pyruvates in den Tumorzellen zu erhalten, wurde der Versuch mit

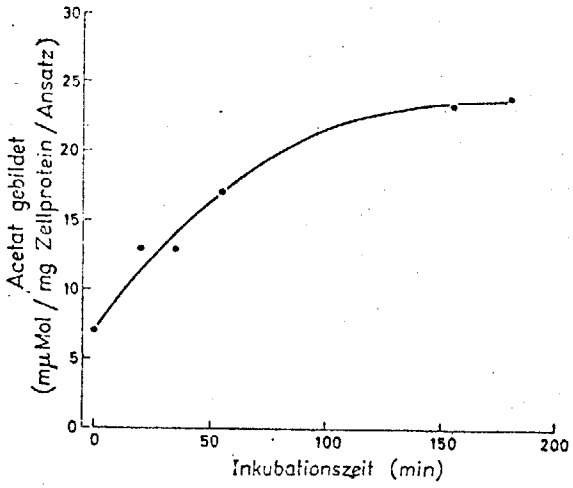


Fig. 4. *Acetatbildung von Ascites-Tumorzellen.* Gewaschene Tumorzellen (MAT I), entsprechend 70 mg Zellprotein pro Ansatz, wurden ohne Substratzusatz unter Schütteln in Krebs-Ringer-Bicarbonat unter 95% O_2– 5% CO_2 inkubiert. Endvolumen 3,0 ml, $t = 37°$ C. Die Ansätze wurden zu den angegebenen Zeiten durch Zusatz von Perchlorsäure (Endkonzentration 3%) gestoppt. In den klarzentrifugierten Filtraten wurde die Essigsäure bestimmt (vgl. Methodik)

2-^{14}C-markiertem Pyruvat wiederholt (Fig. 6). In diesem Versuch erscheinen rund 20% des Pyruvates als Acetat, etwa 45% wurden als $^{14}CO_2$ erfaßt, das aus der Endoxydation via Citronensäurecyclus stammen muß.

Wie in Fig. 5 und 6 auffällt, kommt es nach Pyruvatzusatz zu

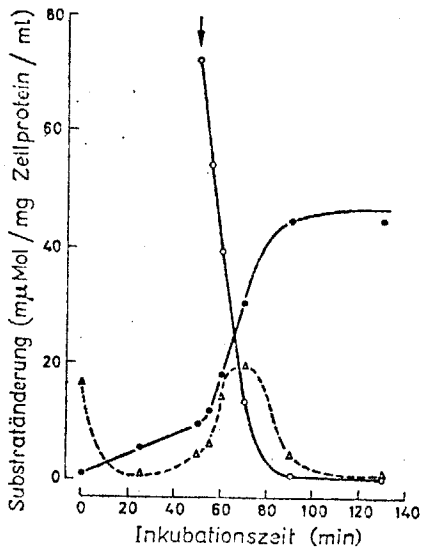


Tabelle 1. *Abhängigkeit der Acetatbildung von der Pyruvatkonzentration*
Gewaschene Tumorzellen (MAT I) wurden wie in Versuch Fig. 4 mit steigenden Mengen von Pyruvat inkubiert. Versuchsdauer: 90 min

Pyruvatkonzentration	Acetat gebildet mμMol/ml pro mg Zellprotein
$2 \cdot 10^{-3}$ M	49,5
$1 \cdot 10^{-2}$ M	82
$2 \cdot 10^{-2}$ M	106,5

Fig. 5. *Stimulierung der Acetatbildung von Ascites-Tumorzellen durch Pyruvat.* Gewaschene Tumorzellen (MAT I), entsprechend 40 mg Zellprotein pro ml Inkubationsmedium, wurden in Krebs-Ringer-Bicarbonat unter konstanter Begasung mit 95% O_2– 5% CO_2 in einem Erlenmeyer-Kolben inkubiert. Zur Substratbestimmung wurden aliquote Teile der Zellsuspension zu den angegebenen Zeiten entnommen und in vorgekühlte Perchlorsäure eingebracht. Nach 50 min (Pfeil) Zugabe von 2,9 μMol Pyruvat pro ml. $t = 37°$ C. Acetat •——•; Pyruvat o——o; Lactat Δ----Δ

einer vorübergehenden Anhäufung von Lactat. Offenbar sind die Tumorzellen nicht in der Lage, die im Überschuß angebotenen Mengen an Pyruvat mit genügender Schnelligkeit zu oxydieren, so daß ein Teil — nach Art eines Überlaufes — zunächst in Lactat umgewandelt wird. Diese Erscheinung entspricht der bekannten hohen aeroben Glykolyse der Ascites-Tumorzellen [41].

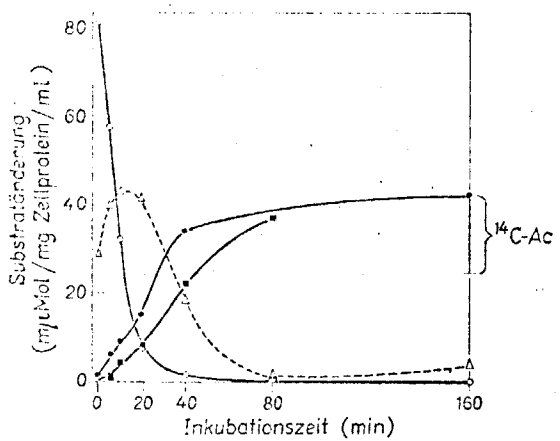


Fig. 6. *Abbau von 2-^{14}C-Pyruvat durch Ascites-Tumorzellen.* Gewaschene Tumorzellen (MAT I), entsprechend 40 mg Zellprotein pro ml, wurden in Einzelansätzen in Krebs-Ringer-Bicarbonat unter 95% O_2 – 5% CO_2 inkubiert. Stoppen der Reaktion mit Perchlorsäure und Auffangen des $^{14}CO_2$ mit Phenylathylamin erfolgte in üblicher Weise durch Injektion in das geschlossene Reaktionsgefäß. Zur Bestimmung der nach 160 min im Acetat vorliegenden Radioaktivität wurde der Ansatz der Mikrodiffusion unterworfen. In der Abbildung ist der aus Pyruvat entstandene Essigsäureanteil (^{14}C-Ac) eingezeichnet. Unter diesen Versuchsbedingungen geht ca. 1% der gebildeten radioaktiven Essigsäure mit dem $^{14}CO_2$ in das Phenylätlylamin über. Zu Versuchsbeginn Zugabe von 3,26 μMol 2-^{14}C-Pyruvat pro ml, t = 37° C. Weitere Behandlung vgl. Versuch Fig. 4. Acetat ●——●; Pyruvat ○——○; Lactat △- - - -△; $^{14}CO_2$ ■——■

Inkubiert man die Ehrlich-Zellen unter Stickstoff, so findet durch Pyruvat keine über die Leerproduktion hinausgehende Acetatbildung statt. Wie aus Fig. 7 hervorgeht, wird unter anaeroben Bedingungen das gesamte Pyruvat in Lactat umgewandelt. Aus diesem Versuch kann geschlossen werden, daß Acetat nicht auf dem Wege einer Dismutationsreaktion gemäß

$$2 \text{ Pyruvat} \rightarrow 1 \text{ Acetat} + 1 \text{ Lactat} + 1 \text{ CO}_2$$

entsteht, wie dies in anderen Geweben beobachtet wurde [23].

Mechanismus der aeroben Umwandlung von Pyruvat in Acetat. Nach Untersuchungen Holzers et al. findet in Hefezellen eine Coenzym A-unabhängige Umwandlung von Pyruvat in Acetat statt [20]. Bei dieser im Cytoplasma der Hefezellen ablaufenden Reaktion wird Acetaldehyd aus Hydroxyäthyl-thiaminpyrophosphat freigesetzt und zur Essigsäure

Tabelle 2

Wirkung von Arsenit auf die Pyruvatdekarboxylierung von Ascites-Tumorzellen

Inkubation gewaschener Tumorzellen (MAT I) in Krebs-Ringer-Phosphat unter Luft mit 4,32 μMol 1-^{14}C-Pyruvat. Endvolumen 2,0 ml, t = 37° C. Stoppen der Reaktion und Auffangen des gebildeten $^{14}CO_2$ erfolgte in üblicher Weise [41]. Bestimmung des Pyruvates optisch-enzymatisch [7]

Arsenitkonzentr. (Mol/l)	$^{14}CO_2$ (in Prozent der einges. Imp.)	Verbliebenes nicht flüchtiges ^{14}C (Prozent der einges. Imp.)	Verbliebenes Pyruvat (enzym.) (Prozent der einges. Menge)
0,0	30	65	13
$1{,}2 \cdot 10^{-4}$	2,72	91,7	82
$2{,}4 \cdot 10^{-4}$	2,11	93	94
$2{,}0 \cdot 10^{-3}$	0,47	84	84

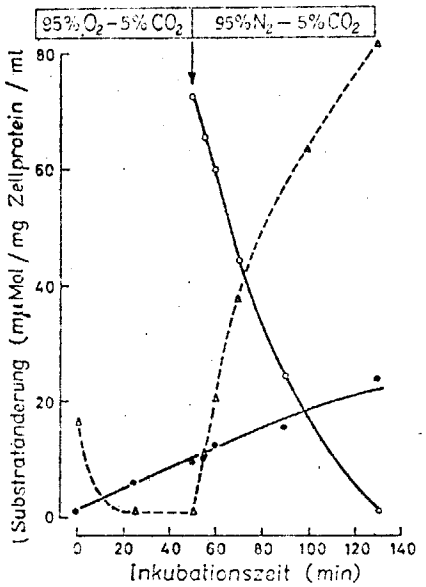


Fig. 7

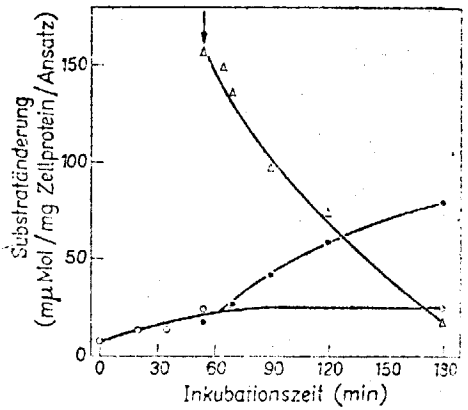


Fig. 8

Fig. 7. *Ausbleiben der pyruvatabhängigen Acetatbildung unter anaeroben Bedingungen.* Versuchsanordnung vgl. Fig. 5. Nach 50 min Wechsel der Gasphase zu 95% N_2 – 5% CO_2. Acetat ●——●; Pyruvat ○——○; Lactat △- - - -△

Fig. 8. *Acetatbildung von Ascites-Tumorzellen aus Acetaldehyd.* Gewaschene Tumorzellen (MAT I), entsprechend 70 mg Zellprotein pro Ansatz wurden in Krebs-Ringer-Bicarbonat unter 95% O_2 – 5% CO_2 inkubiert. Zugabe von 4,15 μMol Acetaldehyd nach 55 min (Pfeil). Endvolumen 3,06 ml. Weiterverarbeitung wie bei Versuch Fig. 4. Acetat ●——●; Acetat ohne Substrat ○——○; Acetaldehyd △——△

oxydiert. Diese erscheint in Hefe allerdings nicht als Endprodukt, da sie rasch durch die aktive Acetat-thiokinase aktiviert wird.

Um zu prüfen, ob ein ähnlicher Weg der Acetatbildung auch in den Asciteszellen in Betracht kommt, versuchten wir, den liponsäureabhängigen Schritt Hydroxyäthyl-thiaminpyrophosphat → Acetyl-CoA durch Arsenit zu blockieren [21, 32, 35], um damit möglicherweise die Essig-

säurebildung zu stimulieren. Bei Einsatz 1-^{14}C-markierten Pyruvates stellte sich jedoch heraus, daß bereits die Decarboxylierung durch Arsenit gehemmt wird (Tab. 2). Somit war es also nicht möglich, die Frage einer CoA-unabhängigen Acetatproduktion aus Pyruvat durch Einsatz von Arsenit weiter zu klären.

Wir versuchten deshalb, direkt die Bildung von Acetaldehyd aus Pyruvat nachzuweisen. Bei Inkubation von Ascitestumorzellen mit Pyruvat (Konzentration 0,0033 und 0,01 M) unter aeroben Bedingungen

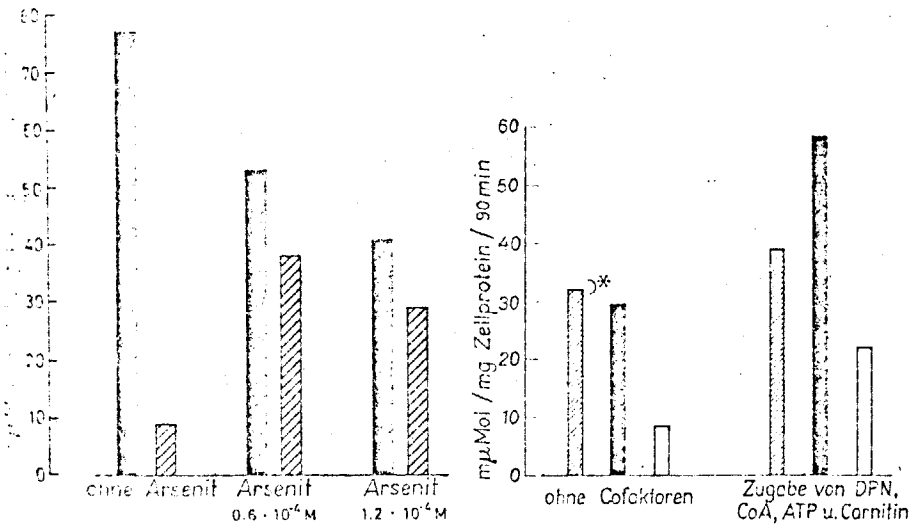


Fig. 9 Fig. 10

Fig. 9. *Acetatbildung von Ascites-Tumorzellen aus Pyruvat in Anwesenheit von Arsenit.* Gewaschene Tumorzellen (MAT I), entsprechend 55 mg Zellprotein pro Ansatz wurden mit 0,01 M Pyruvat in Krebs-Ringer-Phosphat unter Luft inkubiert. Endvolumen 3,0 ml, t = 37° C. Die Ansätze wurden nach 90 min mit Perchlorsäure gestoppt. ▨ Pyruvat 0,01 M; ▨ kein Substrat

Fig. 10. *Acetat- und CO_2-Bildung von Ascites-Tumorzellen mit Palmitat als Vorläufer. Wirkung von Cofaktoren.* Gewaschene Tumorzellen (MAT I), entsprechend 35 mg Zellprotein pro Ansatz, wurden in Krebs-Ringer-Bicarbonat unter 95% O_2 + 5% CO_2 mit 0,001 M 1-^{14}C-Palmitat-Albuminkomplex([13]) 90 min inkubiert. ▨ CO_2; ▨ Acetat; ▨ Acetat ohne Substratzusatz. Der mit * gekennzeichnete CO_2-Wert stammt aus einem anderen Versuch. Hier war Palmitat 0,00033 M

ließ sich optisch-enzymatisch kein freier Acetaldehyd nachweisen. Auf der anderen Seite vermögen Asciteszellen aus Acetaldehyd Essigsäure zu bilden (Fig. 8).

In den Arsenit-Hemmungsversuchen ergab sich erwartungsgemäß eine zunehmende Unterdrückung der Essigsäurebildung aus Pyruvat (Fig. 9). Gleichzeitig fiel jedoch auf, daß die Tumorzellen ohne Substratzusatz in Anwesenheit von Arsenit mehr Acetat produzierten, als wenn sie ohne Arsenit inkubiert wurden. Dieses Extraacetat muß demnach

aus endogenen Acetatvorstufen entstanden sein, vermutlich denselben, die auch für die Acetat-„Leerbildung" in den vorausgehenden Versuchen (Fig. 4) verantwortlich waren. Unter der Annahme, daß es sich dabei um Fettsäuren handelte, konnte der Arsenitefekt dahingehend gedeutet werden, daß es durch Hemmung des Citronensäurecyclus auf der Stufe des α-Ketoglutarates zu einem Anstau von Acetyl-CoA kommt, und daß die Essigsäure durch enzymatische Spaltung des Acetyl-CoA entsteht. Diese Beobachtung veranlaßte weitere Untersuchungen mit dem Ziel, die Acetatbildung der Tumorzellen durch langkettige Fettsäuren zu stimulieren.

Acetatbildung aus Fettsäuren. Fig. 10 läßt erkennen, daß die Acetatbildung der Asciteszellen — ähnlich wie durch Pyruvat — auch durch Zusatz langkettiger Fettsäuren gesteigert wird. In diesen Versuchen wurde 1-^{14}C-Palmitat in Form eines Albuminkomplexes, bereitet nach [13], angeboten. Man sieht aus diesen Versuchen außerdem, daß optimale Fettsäureoxydation nur nach Zugabe der Cofaktoren CoA, ATP und DPN vonstatten geht. Carnitin bewirkt — wie auch in anderen Geweben [15, 16] — eine zusätzliche Stimulierung, sowohl der $^{14}CO_2$-Bildung als auch der Essigsäurebildung. Es erscheint bemerkenswert, daß die genannten Cofaktoren auch die Acetatproduktion aus endogenen Quellen stimulieren.

Wie in den Versuchen ohne Substratzusatz (Fig. 9) wurde die Essigsäurebildung der Tumorzellen aus Palmitat ebenfalls durch Arsenit gesteigert. Mit Fluoracetat [4, 33] fanden wir dagegen weder eine Hemmung der $^{14}CO_2$-Bildung noch eine Steigerung der Acetatbildung. Dies erklärt sich aus der Tatsache, daß Fluoracetat in den Asciteszellen nur mangelhaft aktiviert wird und deshalb die erforderlichen Hemmkonzentrationen an Fluorcitrat offenbar nicht erreicht werden (siehe auch [33, 34]).

Vergleich der Acetatproduktion verschiedener Tumoren. Die Nettobildung freier Essigsäure ist nicht eine spezielle Eigenschaft des in den bisher beschriebenen Untersuchungen verwendeten Mäuseascites-Tumors. Wie Fig. 11 zeigt, produzieren auch andere Ascitestumorzellen, sowohl bei Inkubation ohne Substrat als auch mit Pyruvat oder Palmitat, wechselnde Mengen an Acetat. Bemerkenswert erscheint der Befund der Acetatbildung aus Pyruvat durch Dünnschnitte eines soliden, transplantablen Hepatoms[4], der in Fig. 12 dargestellt ist. Unter identischen Versuchsbedingungen setzen Dünnschnitte normaler Ratten- und Mäuselebern keine Essigsäure in Freiheit.

[4] Transplantables DANA-Lebercarcinom der Ratte. Für die Überlassung des Tumors sind wir Herrn Prof. Dr. H. Druckrey, Forschergruppe für Präventivmedizin, Freiburg, zu großem Dank verpflichtet.

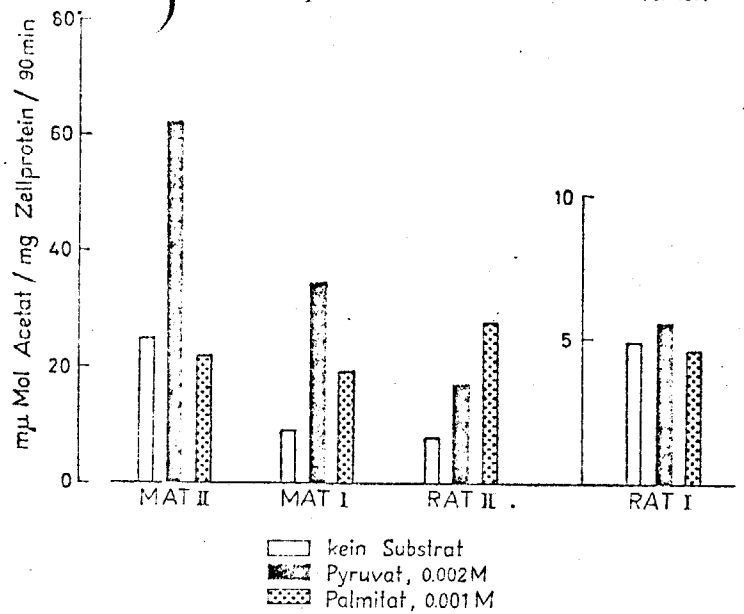


Fig. 11. *Acetatbildung verschiedener Tumoren aus Pyruvat und Palmitat.* Bedingungen analog zu Versuchen Fig. 9 und 10

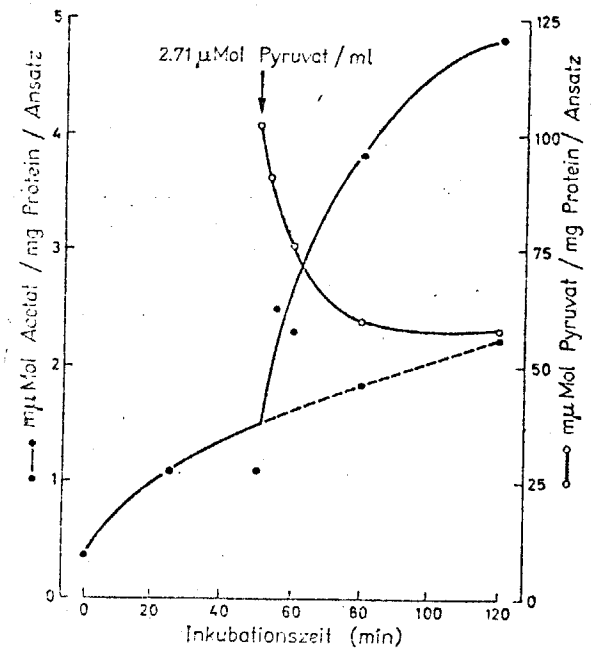


Fig. 12. *Stimulierung der Acetatbildung durch Pyruvat in Dünnschnitten eines Hepatocarcinoms.* Dünnschnitte eines DÄNA-Lebercarcinoms (vgl. Fußnote S. 96) entsprechend 300 mg Feuchtgewicht, wurden in Krebs-Ringer-Bicarbonat in Einzelansätzen von 3,0 ml Endvolumen unter 95% O_2 – 5% CO_2 inkubiert. Zusatz von 2,71 μMol Pyruvat pro ml nach 50 min (Pfeil). $t = 37°$ C. Weiterverarbeitung der Proben analog zu Versuch Fig. 4. Die gestrichelte Kurve gibt die Acetatbildung in Abwesenheit von Pyruvat wieder

Gehalt an Coenzym-A-Derivaten. Acetyl-CoA konnten wir in $HClO_4$-Extrakten aus Lyophilisaten von MAT I mit dem Test nach Ochoa [40,43] nicht nachweisen. Der Gehalt an freiem CoA betrug 40 mμMol pro Gramm Trockengewicht gegenüber 27,8 mμMol an säureunlöslichem CoA pro Gramm Trockengewicht. Die gleiche Tumorpassage enthielt 6,16 μMol Acetat pro Gramm Trockengewicht.

Versuche mit Tumorextrakten. Nach den mit intakten Zellen erhaltenen Ergebnissen bot sich als nächstliegende Erklärung für das Zustandekommen der Acetat-Nettobildung ein Mißverhältnis zwischen

Tabelle 3. *Spezifische Aktivitäten und Michaelis-Konstanten der Acetat-thiokinase und der Acetyl-CoA-deacylase in Leber- und Tumorextrakten*

		Thiokinase[1]	Deacylase[1]	Deacylase / Thiokinase
Mäuseleber	spez. Akt.	6,7	9,6	1,43
	K_M	$1{,}8 \cdot 10^{-4}$ M (Acetat)	$0{,}6 \cdot 10^{-4}$ M (Acetyl-CoA)	
Ehrlich-Ascites-Carcinom	spez. Akt.	0,11	7,05	64
	K_M	$1{,}6 \cdot 10^{-4}$ M (Acetat)	$0{,}3 \cdot 10^{-4}$ M (Acetyl-CoA)	
DÄNA-Leber-Carcinom	spez. Akt.	0,36	3,5	9,7

[1] Enzym-Bestimmung bei 37° C. — [2] Enzym-Bestimmung bei 25° C.

Acetataktivierung und Acetyl-CoA Spaltung an. Diese Vorstellung ließ sich experimentell durch Aktivitätsbestimmungen der Enzyme Acetat-thiokinase und Acetyl-CoA-deacylase unterbauen. Tatsächlich ist — wie aus Tab. 3 hervorgeht — die Aktivität der Thiokinase in den Tumorzellen äußerst schwach und macht nur etwa den 60. Teil der Aktivität der Leber aus. Dagegen liegt die Aktivität der Deacylase in Tumor und Leber etwa gleich hoch. Das Verhältnis Deacylase : Thiokinase beträgt in der Leber etwa 1,4, im MAT I-Tumor dagegen 64. Wie Tab. 3 zeigt, ergibt sich ähnlich niedrige Thiokinaseaktivität nicht nur in Ascitestumorzellen, sondern auch in dem von uns untersuchten Rattenhepatom. Um zu prüfen, ob neben diesen quantitativen Unterschieden auch qualitative Veränderungen der genannten Enzyme vorliegen, bestimmten wir ihre Affinität gegenüber Acetat bzw. Acetyl-CoA. Nach den Substratsättigungskurven (Fig. 13 und 14) ergeben sich jedoch keine nennenswerten Unterschiede. So liegt die Michaeliskonstante der Thiokinase aus Leber bei $1{,}8 \cdot 10^{-4}$ M Acetat, der Thiokinase aus Ascitestumor bei $1{,}6 \cdot 10^{-4}$ M Acetat. Für die Deacylase aus Leber beträgt $K_m = 0{,}6 \cdot 10^{-4}$ M Acetyl-CoA, für diejenige aus Tumor $0{,}3 \cdot 10^{-4}$ M Acetyl-CoA.

331

Diskussion

Die hier beschriebenen Untersuchungen werfen als erstes die Frage auf, ob man die aerobe Produktion freier Essigsäure durch Ascitestumorzellen — ähnlich wie die hohe aerobe Glykolyse — als eine allgemeine Eigenart maligner Geschwülste auffassen darf, oder ob sie nur einen Spezialfall bestimmter Tumoren darstellt. In der vorliegenden

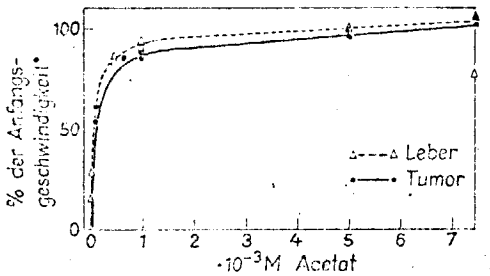


Fig. 13. *Substratsättigungskurve der Acetat-thiokinase. Vergleich von Leber und Tumor.* Zusammensetzung des Tests siehe Methodik. Mäuseleber Δ----Δ; MAT I •—— •

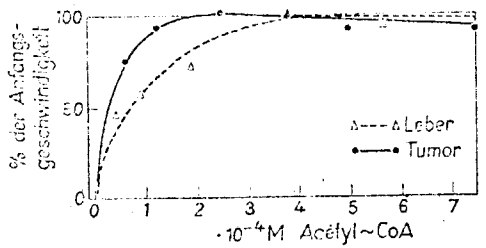


Fig. 14. *Substratsättigungskurve der Acetyl-CoA-deacylase. Vergleich von Leber und Tumor.* Zusammensetzung des Tests siehe Methodik. Mäuseleber Δ----Δ; MAT I •——•

Arbeit konnte dieses Merkmal bei vier verschiedenen Ascitestumoren — davon zwei Carcinome, ein Sarkom, ein Hepatom — und bei einem soliden Hepatom beobachtet werden. Während unsere Untersuchungen in vollem Gange waren, nahmen wir mit besonderem Interesse eine Mitteilung F. LYNENS zur Kenntnis, der schon 1940 die Bildung von Acetat in Schnitten des Jensen-Sarkoms der Ratte gefunden hatte [26]. Zur gleichen Zeit wurde von ELLIOT et al. [12] die Vermutung ausgesprochen, daß bei der Oxydation von Pyruvat durch verschiedene Tumoren (Philadelphia No. 1 Sarcom, Walker 256 Carcinom) Essigsäure auftritt, jedoch konnte der experimentelle Beweis hierfür nicht beigebracht werden. Auch FREEDMAN u. GRAFF [14] schlossen aus ihren Isotopen-Verteilungsstudien auf die Bildung eines C-2-Körpers aus Pyruvat in Dünnschnitten eines Murphy-Sturm-Lymphosarkoms.

Auf die Frage, wie es zur Nettoproduktion von Acetat in den Tumorzellen kommt, scheinen unsere Messungen der Enzyme Acetat-thiokinase

und Acetyl-CoA-deacylase eine plausible Antwort zu geben. Man kann sich vorstellen, daß Thiokinase und Deacylase in normalen Geweben so aufeinander abgestimmt sind, daß keine Freisetzung von Essigsäure stattfindet (Fig. 15). Im Tumor ist jedoch die Aktivität der Thiokinase besonders schwach im Verhältnis zur Deacylase (vgl. Tab. 3), so daß die Acetataktivierung mit der Acetyl-CoA-Spaltung nicht mehr Schritt hält. Das Ergebnis ist die Nettobildung freier Essigsäure.

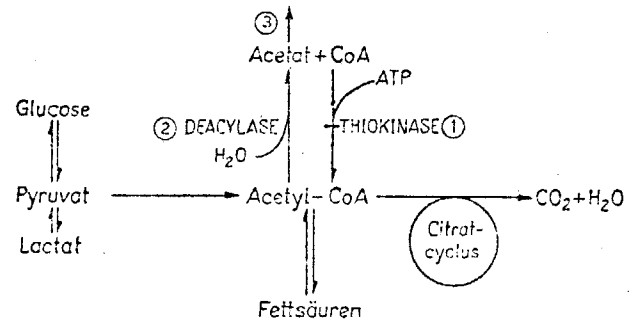


Fig. 15

Mechanismus der Acetatbildung in Tumorzellen. Die Acetataktivierung kann auf Grund der geringen Thiokinaseaktivität der Tumorzellen ① mit der Acetyl-CoA-Spaltung ② nicht Schritt halten. Dadurch kommt es zur Unterbrechung des Acetatkreislaufes und zur Nettobildung freier Essigsäure ③

Die schwache Ausstattung der von uns untersuchten Tumoren mit dem Acetat-aktivierenden Enzym bietet auch eine Erklärung für zahlreiche frühere Befunde, die auf die geringe Acetatverwertung einer ganzen Reihe von Tumoren hindeuten. So fand man schon 1935, daß Acetat von Schnitten aus Philadelphia No. 1 Sarcom und Walker 256 Carcinom der Ratte nur schlecht oxydiert wird [11]. Aus Versuchen mit 1-^{14}C-Acetat an Schnitten von Flexner-Jobling Carcinom und Walker 256 Carcinom schlossen PARDEE et al. [31], daß die Aktivierung der Essigsäure — gemessen am $^{14}CO_2$ — nur schwach sei. Dies wurde dann von BUSCH [8, 9] durch in vivo Studien an Tumorratten (Jensen, Walker 256, Flexner-Jobling, Lymphom) bestätigt. Nach Injektion von 1-^{14}C-Acetat blieb in den Tumoren der Großteil der Radioaktivität als Essigsäure liegen, während in anderen Geweben 70—100% der Aktivität in der gleichen Zeit in nichtflüchtige Substanzen überging. Mit und ohne Malonat-Vorbehandlung zeigte sich eine sehr geringe $^{14}CO_2$-Bildung aus Acetat, die die Autoren zu der Annahme bestimmte, daß Tumoren praktisch keine Essigsäure verwerten. In dieselbe Richtung weisen Beobachtungen von MEDES et al. [27], wonach Dünnschnitte verschiedener Tumoren auffallend wenig Acetat in Lipide einzubauen vermögen.

Die Vorstellung, daß in der Tumorzelle ständig ein Teil des Acetyl-CoA hydrolysiert und damit dem Citronensäurecyclus entzogen wird,

läßt auch causale Überlegungen im Hinblick auf die bekannte hohe aerobe Glykolyse maligner Zellen zu [41] (Übersicht vgl. [1]). Tatsächlich würde der laufende Verschleiß von Acetyl-CoA zu einem Mangel an Atmungsenergie führen, ein Zustand, der kompensatorisch zur Ankurbelung der aeroben Glykolyse Veranlassung geben könnte. Die primäre Störung läge also in diesem Falle in einem umschriebenen Enzymdefekt — eben in der Verarmung der Tumorzellen an Acetatthiokinase. Zur weiteren Klärung dieser Frage erscheint es notwendig, die Studien auf eine möglichst große Anzahl verschiedenartiger Tumoren auszudehnen und vor allem auch Tumoren geringster Abweichung, wie z. B. das Morris Hepatom 5123 [29] und andere in die Untersuchungen miteinzubeziehen.

Von SIPERSTEIN u. FAGAN [36] liegen einige Daten über den Acetatstoffwechsel in Hepatomen vor. Daraus geht hervor, daß die $^{14}CO_2$-Bildung im Vergleich zu normaler Leber stark eingeschränkt ist, während der Acetateinbau in die Fettsäuren und das Cholesterin in den Tumoren sogar gesteigert ist. Diese Befunde würden nicht für eine Störung der Acetat-Aktivierung sprechen, doch erscheinen genauere enzymatische Untersuchungen erforderlich.

Der Deutschen Forschungsgemeinschaft, Bad Godesberg, dem Bayerischen Staatsministerium für Unterricht und Kultus und den Farbwerken Hoechst, Frankfurt-Höchst, sind wir für die großzügige Unterstützung der vorliegenden Arbeiten zu großem Dank verpflichtet.

Literatur

[1] AISENBERG, A. C.: The Glycolysis and Respiration of Tumors. New York and London: Academic Press 1961.
[2] EISENHERZ, G., H. I. BOLTZE, TH. BÜCHER, R. CZOCK, K. H. GARBADE, E. MEYER-ARENDT u. G. PFLEIDERER: Z. Naturforsch. 8b, 555 (1953).
[3] BERGMEYER, H. U.: In: Methoden der enzymatischen Analyse, S. 290. Weinheim: Verlag Chemie 1962.
[4] BRADY, R. O.: J. biol. Chem. 217, 213 (1955).
[5] BRAY, G. A.: Analyt. Biochem. 1, 279 (1960).
[6] BUCKEL, W., u. H. EGGERER: Biochem. Z. 343, 29 (1965).
[7] BÜCHER, TH., R. CZOCK, W. LAMPRECHT u. E. LATZKO: In: Methoden der enzymatischen Analyse, S. 253. Weinheim: Verlag Chemie 1962.
[8] BUSCH, H.: Cancer Res. 13, 789 (1953).
[9] —, and H. A. BALTRUSH: Cancer Res. 14, 448 (1954).
[10] EGGERER, H.: Pers. Mitteilung.
[11] ELLIOTT, K. A. C., M. P. BENOY, and Z. BAKER: Biochem. J. 29, 1937 (1935).
[12] —, and M. E. GREIG: Biochem. J. 31, 1021 (1937).
[13] FILLERUP, D. L., I. C. MCGHEE, and J. F. MEAD: J. biol. Chem. 233, 98 (1958).
[14] FRIEDMAN, A. D., and S. GRAFF: J. biol. Chem. 233, 292 (1958).
[15] FRITZ, I. B.: Physiol. Rev. 41, 52 (1961).
[16] —, and K. R. N. YUE: J. Lipid Res. 4, 279 (1963).
[17] GERCHELY, J., P. HILLE, and C. V. RAMAKRISHNAN: J. biol. Chem. 198, 323 (1952).

[18] HOCHHEUSER, W., H. WEISS u. O. WIELAND: Z. klin. Chem. 2, 175 (1964).
[19] HOHORST, H. J.: In: Methoden der enzym. Analyse, S. 266. Weinheim: Verlag Chemie 1962.
[20] HOLZER, H., u. H. W. GOEDDE: Biochem. Z. 329, 175 (1957).
[21] — — Biochem. Z. 329, 192 (1957).
[22] JONES, M. E., S. BLACK, R. M. FLYNN, and F. LIPMANN: Biochim. biophys. Acta (Amst.) 12, 141 (1953).
[23] KREBS, H. A., and W. A. JOHNSON: Biochem. J. 31, 645 (1937).
[24] LUNDQUIST, F., U. FUGMANN, E. KLÄNING, and H. RASMUSSEN: Biochem. J. 72, 409 (1959).
[25] LYNEN, F., P. A. SRERE, and W. SEUBERT: Biochim. biophys. Acta (Amst.) 33, 313 (1959).
[26] — Unveröffentlicht.
[27] MEDES, G., A. J. THOMAS, and S. WEINHOUSE: Cancer Res. 13, 27 (1953).
[28] MELLANBY, J., u. D. H. WILLIAMSON: In: Methoden der enzymatischen Analyse, S. 454. Weinheim: Verlag Chemie 1962.
[29] MORRIS, H. P., SIDRANSKY, H., B. P. WAGNER, and H. M. DYER: Cancer Res. 20, 1252 (1960).
[30] NUMA, S., M. MATSUHASHI u. F. LYNEN: Biochem. Z. 334, 203 (1961).
[31] PARDEE, A. B., C. HEIDELBERGER, and V. R. POTTER: J. biol. Chem. 186, 625 (1950).
[32] PETERS, R. A., H. M. SINCLAIR, and R. H. S. THOMPSON: Biochem. J. 40, 516 (1946).
[33] POTTER, V. R., and H. BUSCH: Cancer Res. 10, 353 (1950).
[34] REISS, O. K., L. HELLERMANN, J. A. AMBROSE, C. I. SMITH, M. K. GEY, and A. E. IBER: J. biol. Chem. 237, 992 (1962).
[35] SCHREIBER, G., G. KOHLHAW, H. W. GOEDDE u. H. HOLZER: Biochem. Z. 339, 83 (1963).
[36] SIPERSTEIN, M. D., and V. M. FAGAN: Cancer Res. 24, 1108 (1964).
[37] SIMON, E. J., and D. SHEMIN: J. Amer. chem. Soc. 75, 2520 (1953).
[38] SOODAK, M., and F. LIPMANN: Fed. Proc. 7, 190 (1948).
[39] STADTMAN, E. R.: Aus: MICHAL, G., u. H. U. BERGMEYER. In: Methoden der enzymatischen Analyse, S. 517. Weinheim: Verlag Chemie 1962.
[40] STERN, I. R., B. SHAPIRO, E. R. STADTMAN, and S. OCHOA: J. biol. Chem. 193, 703 (1951).
[41] WARBURG, O.: Über den Stoffwechsel der Tumoren. Berlin: Springer 1926.
[42] WIELAND, O., u. I. NEUFELDT: Hoppe-Seylers Z. physiol. Chem. 324, 101 (1961).
[43] —, u. L. WEISS: Biochem. biophys. Res. Commun. 10, 333 (1963).
[44] — L. WEISS u. I. EGER-NEUFELDT: Biochem. Z. 339, 501 (1964).
[45] WRBA, H., u. H. RABES: Z. Krebsforsch. 65, 316 (1963).
[46] ZAJDELA, F.: Colloque Franco-Sovietique, Moscou, Julliet 1962. p. 49. Paris: Gauthier-Villars 1963.

Prof. Dr. O. WIELAND
Klinisch-chemisches Institut des Städt.
Krankenhauses München-Schwabing
8 München, Kölner Platz 1

331

Absorption of Acetic Acid and Glycerol From the Rat Stomach[1, 2]

DAVID C. HERTING, NORRIS D. EMBREE AND PHILIP L. HARRIS

From the Research Laboratories, Distillation Products Industries, Division of Eastman Kodak Company, Rochester, New York

Amer. J. Physiol 187(2): 224-226, (1956)

ABSTRACT

The absorption of acetic acid from the pylorus-ligated rat stomach in 6 hours showed a typical log-dose response, with 2.0 mM being absorbed at the highest level administered. Under the same conditions, the amount of glycerol absorbed reached and maintained a constant level approximating 0.4 mM. The gastric contents of rats consuming a diet containing 30% of distilled, saturated, partially acetylated monoglycerides contained an average of 5.6 mg (0.09 mM) of free acetic acid/rat.

THE LIPID MATERIAL recovered from gastric contents of rats fed fully saturated fats, such as hydrogenated lard and distilled saturated acetylated monoglycerides, contained high levels of free, long-chain fatty acids (1). To measure any free acetic acid which might have come from the acetylated monoglycerides in the diet, an analytical technique was developed. Since only small amounts were found, a study was made of the absorption of acetic acid from the stomach. The gastric absorption of glycerol was also investigated.

METHODS

Adult albino rats of the Sprague-Dawley strain were housed individually and were fed a diet containing 30% of saturated, partially acetylated monoglycerides as described previously (1). To obtain stomach contents, the rats were anesthetized with ether, an incision was made, and hemostats were placed at the cardiac and pyloric sphincters. The stomachs were removed, cut open and rinsed with distilled water. Acetic acid was measured immediately, using either the entire contents or a filtrate of the contents after chilling.

For the absorption studies, pylorus ligated rats were prepared as described by Herting and Steenbock (2). Aqueous solutions of acetic acid or glycerol were placed in the stomach by inserting a hypodermic needle into the proximal portion of the duodenum, through the pylorus, and into the stomach. A ligature around the pyloric sphincter was tightened, the solution was injected, and the needle was withdrawn. After the desired time the stomach was emptied and was rinsed several times with distilled water. The ability to recover standard solutions from the stomach was measured with zero hour animals. In these studies, 4 or 5 ml of distilled water were injected into the stomach to dilute the acetic acid or glycerol before emptying for the first time. In addition, each experiment included several control rats. The gastric contents of these rats were analyzed for both steam-distillable acidity and periodate-oxidized materials. The results served as a base line for the experimental animals.

Glycerol was determined by oxidation with periodic acid essentially as described by Handschumaker and Linteris (3). No fat solvent was necessary and an 11-minute oxidation period was used. Acetic acid was determined by modifying a technique suggested by the Cellulose Acetate Development Division, Eastman Kodak Company.[3] The solution was diluted to 100 ml and was adjusted to about pH 4, using methyl orange indicator. A 5-ml aliquot of aqueous $AgNO_3$ (15% w/v) was added to prevent high recoveries due to distillation of gastric HCl. Under the same conditions, the HNO_3 formed did not distill. The solution was steam-distilled to dryness at reduced pressure (water pump) with the distilling flask in a bath at 65°C and the receiving flask in chilled brine. Bumping was prevented by adding several drops of Dow-Corning antifoam agent A and by air bubbling into the system through a capillary tube immersed in the distilling solution. Two 100-ml portions of distilled water were added separately to the distilling flask and steam distilled to dryness at reduced pressure. A portion of the combined distillates was titrated to a phenolphthalein end point with standard NaOH. The distillates were always tested qualitatively to insure the absence of chloride.

RESULTS

The quantitative aspects of the analytical technique for acetic acid are shown in table 1.

Received for publication June 8, 1956.

[1] Communication No. 231 from the Research Laboratories, Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N. Y.

[2] Presented in part at the meetings of the Federation of American Societies for Experimental Biology, April 16-20, 1956, Atlantic City, N. J.

[3] We express our appreciation to Dr. Leo B. Genung for suggesting the basic technique.

336

The total titrable acidity of the distillate from 2 gm of stock diet plus acetic acid was corrected for the acidity of the distillate from stock diet alone. Recoveries were excellent in all trials, averaging 102.7 ± 1.9% S.D.

When this technique was applied to stomach contents of rats consuming a diet containing 30% of saturated, partially acetylated monoglycerides, seven groups of three rats each contained from 1.4–13.1 mg of acetic acid/rat, with an average of 5.6 ± 1.5 mg S.E./rat. The identity of the acetic acid was checked by chromatography of the hydroxamate derivative on Whatman no. 1 paper, using the alcohol phase of a system containing amyl alcohol 3:formic acid 1:H_2O 3 as the mobile solvent (4).

These small amounts of free acetic acid in the stomach indicated either that it did not appear in sizeable amounts in the stomach or that it was removed rapidly via passage into the small intestine or via absorption from the stomach. Investigation of the latter possibility with pylorus-ligated rats revealed that significant amounts of acetic acid can be absorbed from the pylorus-ligated stomach (fig. 1).

The absorption of acetic acid during a 6-hour period showed a typical log-dose response within the range tested. A total of 12 rats was used for these studies. The average recovery of acetic acid at zero hours from 12 rats was 96.8 ± 7.0% S.D. The recovery at zero hours after injecting 421 mg averaged 91%, indicating an immediate uptake or binding of some acetic acid by the gastric mucosa when concentrated solutions were administered.

The absorption of glycerol from the stomach was less marked during the 6-hour period (fig. 1). No absorption occurred when up to 40 mg was injected. At a dose of 115 mg, about 37 mg was absorbed. Increasing the dose up to 405 mg did not increase the amount absorbed. A total of 14 rats was used in this study. The average recovery of glycerol at zero hours from 14 rats was 94.7 ± 3.8% S.D. The glycerol[1] analyzed 99.0 ± 3.5% S.D. by the analytical technique employed (3).

Data obtained at the end of 18 hours showed extensive absorption of both compounds but were of limited value due to extreme thinning of the mucosa, accompanied by hemorrhaging and ulcer formation.

TABLE 1. RECOVERY OF ACETIC ACID ADDED TO STOCK DIET*

Trial No.	Acetic Acid Added	Titratable Acidity of Distillate (Calc. as Acetic Acid)	Recovery of Added Acetic Acid	
	mg	*mg*	*mg*	%
1	0	1.2		
2	0	1.5		
3	0	1.5		
4	20.0	22.9	21.5	104.4
5	20.0	22.4	21.0	101.9
6	41.1	42.7	41.5	100.5
7	41.1	42.5	40.9	99.5
8	51.8	52.7	51.3	99.0
9	51.8	53.1	51.7	99.8
10	207.5	209.8	205.4	99.0
11	411.3	414.3	412.9	100.4
12	0	1.4		
13	48.3	51.7	50.3	104.1
14	0	2.6		
15	96.6	99.2	96.6	100.0
16	96.6	97.8	95.8	99.[illegible]

*Trials 12 and 13 were made on equal amounts of stomach contents from the same group of rats. *Trials 14* and *15* were made on the stomach contents of another group of rats. The recovery in *trial 16* was corrected on the basis of *trials 12* and *14*.

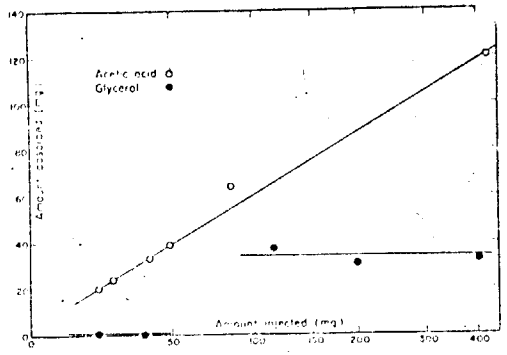


FIG. 1. Absorption of acetic acid and of glycerol from pylorus-ligated rat stomach in 6 hr.

[1] Eastman Organic Chemicals White Label.

DISCUSSION

To our knowledge, the absorption of acetic acid from the stomach of a nonruminant mammal has not been investigated although the gastric absorption of another 2-carbon compound, ethanol, appears to be well established for the dog, rabbit, rat and human being. The only recent review of gastric absorption is that of Faitel'berg (5). The abstract of his review indicates that the absorption of acetic acid is discussed. However, it probably deals with absorption of acetic acid from the rumen, as has been demonstrated by several workers (6, 7). Our results show that significant amounts of acetic acid can be absorbed from the stomach of the rat. At the highest level of injection, about 2.0 mM was absorbed during the 6-hour period.

In contrast, the absorption of glycerol does not appear to be a function of the amount given. A maximum absorption approximating 35 mg for 6 hours is reached and maintained regardless of the amount given. At the highest level of injection this amount corresponds to about 0.38 mM. Thus, the disappearance of glycerol may reflect only passive diffusion whereas acetic acid may be actively absorbed as well. In a previous study, Johnson *et al.* (8) reported a slight absorption of glycerol from the dog stomach. These workers estimated unabsorbed glycerol by measuring the volume of fluid remaining after evaporation of the gastric secretion.

Our results do not establish whether large amounts of free acetic acid are released in the stomach during the digestion of acetylated monoglycerides. The highest level of acetic acid given corresponds to that which could arise from approximately 2.8 gm of the distilled, partially acetylated, saturated monoglycerides which were fed. If gastric hydrolysis of the acetyl-glycerol linkage does occur, it must take place at a rate which permits an immediate and rapid removal of the freed acetic acid from the stomach by direct absorption and by passage into the small intestine. On the other hand, any free glycerol appearing in the stomach would be removed primarily by passage into the intestine.

REFERENCES

1. HERTING, D. C., S. R. AMES, M. KOUKIDES AND P. L. HARRIS. *J. Nutrition* 57: 369, 1955.
2. HERTING, D. C. AND H. STEENBOCK. *J. Nutrition* 57: 400, 1955.
3. HANDSCHUMAKER, E. AND L. LINTERIS. *J. Am. Oil Chem. Soc.* 24: 143, 1947.
4. THOMPSON, A. R. *Australian J. Sci. Research, Ser. B.* 4: 180, 1951.
5. FAITEL'BERG, R. O. *Uspekhi Sovremennoi Biol.* 30: 372, 1950; *Chem. Abstracts* 45: 6719h, 1951.
6. DANIELLI, J. F., M. W. S. HITCHCOCK, R. A. MARSHALL AND A. T. PHILLIPSON. *J. Exper. Biol.* 22: 75, 1945.
7. GRAY, F. V. *J. Exper. Biol.* 25: 135, 1948.
8. JOHNSON, V., A. J. CARLSON AND A. JOHNSON. *Am. J. Physiol.* 103: 517, 1933.

BBA 26348

THE RELATIVE CONTRIBUTION OF ACETATE AND GLUCOSE TO AFLATOXIN BIOSYNTHESIS

D. P. H. HSIEH* AND R. I. MATELES**

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass. 02139 (U.S.A.)

(Received February 6th, 1970)

SUMMARY

1. Fungal secondary metabolites can be classified as acetate-derived or glucose-derived because of the permeability barrier formed by the mitochondrial membrane which separates CoASAc derived from acetate and from glucose. Simultaneous incorporation of acetate and glucose by parallel cultures of *Aspergillus flavus* shows that aflatoxins are acetate-derived metabolites and are probably synthesized extramitochondrially.

2. High levels of exogenous acetate inhibit both glucose utilization and the incorporation of labels from glucose into aflatoxins.

INTRODUCTION

The production of aflatoxins by *Aspergillus flavus* grown in submerged culture[1] has shown that glucose is a good sole carbon source both for growth and for aflatoxin formation. The incorporation of ^{14}C acetate into aflatoxins by resting cell cultures of the mold[2] and the labelling pattern of aflatoxin-B_1 (ref. 3) have further indicated that acetate is a precursor of aflatoxin-B_1. The carbon atoms from glucose and from acetate are channeled into the same product probably through their common metabolic intermediate, CoASAc.

The accumulating experimental evidence for the intracellular localization of enzymes[4,5] suggests that, in cells, such as those of *Aspergillus flavus*, containing mitochondria, the CoASAc derived from glucose and from acetate are found in separate pools. The former is formed by the intramitochondrial oxidation of pyruvate, catalyzed by pyruvate dehydrogenase[6]; whereas the latter arises extramitochondrially through the action of acetate thiokinase, which is found widely in cytoplasm[7,8]. Therefore, the mixing of CoASAc arising from glucose and from acetate involves its transport across the mitochondrial membrane which is rather impermeable to CoASAc. Three mechanisms of the transport of CoASAc across the mitochondrial membrane have been discussed by LOWENSTEIN[9].

* Present address: Department of Environmental Toxicology, University of California, Davis, Calif., U.S.A.

** Present address: Department of Applied Microbiology, Hebrew University-Hadassah Medical School, P.O. Box 1172, Jerusalem, Israel.

Because of the intervention of the mitochondrial membrane as a permeability barrier, determination of the relative rates of incorporation of acetate and glucose into aflatoxins can permit assignment of the pathway into one of the two groups of fungal secondary metabolic pathways. One group consists of the glucose-derivative pathways leading to the biosynthesis of sugar derivatives, glycosides, and shikimic acid metabolites; the other group consists of the acetate-derivative pathways leading to the biosynthesis of polyketides, isoprenoids, and steroids.

This paper describes a parallel culture technique by which the aflatoxin-synthetic pathway was identified as belonging to the acetate-derivative pathway. The technique should be generally useful in the study of fungal secondary metabolism.

MATERIALS AND METHODS

Organism

Aspergillus flavus ATCC 15517 was used throughout this investigation. A conidial suspension in 0.01 % aqueous sodium lauryl sulphate was used to inoculate the growth medium.

Media

The cells were grown on the growth media of ADYE AND MATELES[10]. The composition of the replacement medium used for preparing resting cell cultures was modified from that of ADYE AND MATELES[10] by removing glucose from the formula.

Culture technique

A parallel culture technique was employed to compare the incorporation efficiency of acetate and glucose into aflatoxins. About $2 \cdot 10^5$ conidia were inoculated into 100 ml of the synthetic growth medium contained in a 500-ml baffled erlenmeyer flask and then incubated at 30° with shaking at 200 rev./min. After incubation for 70 h, the mycelial pellets in the flasks were collected by filtration through cheesecloth. They were washed and dispersed twice with distilled water in a Waring blender running at 17 000 rev./min for 10 sec, and again collected on the cheesecloth. Two 5-g portions of the fresh mycelial cake were suspended in two 100-ml portions of the replacement medium each contained in a 500-ml baffled flask. To one flask was added 2000 μmoles of ^{14}C glucose and 500 μmoles of unlabelled acetate; and to the other flask 2000 μmoles of unlabelled glucose and 500 μmoles of 1-^{14}C acetate. Thus, the two flasks were identical except for the labelling. They were incubated at 30° on a rotary shaker shaking at 200 rev./min, and samples from each flask were drawn at time intervals and filtered.

Assays

The residual precursor in the sample broth was determined from the residual radioactivity. A Packard Tri Carb Model 2002 liquid scintillation spectrometer was used to measure the radioactivity. Preliminary experiments showed that substantially all of the radioactivity found in the medium was either labelled precursor (glucose or acetate) or aflatoxins. Aflatoxins were extracted from samples by two extractions with 2 vol. of chloroform, and then purified by thin-layer chromatography[11,12]. The absorbance of the chloroform eluates of aflatoxins from thin-layer chromatograms was

measured at 362 mμ, and converted to concentration of aflatoxins by using a molar extinction coefficient of 20000. The molar quantity of precursor incorporated into aflatoxins was calculated by dividing the radioactivity of the aflatoxin produced by the specific radioactivity of the precursor.

Relative isotope content

To show the precursor-product relationship of a labelled compound with aflatoxins, a relative isotope content was defined as follows: Relative isotope content = (specific radioactivity of aflatoxins recovered)/(specific radioactivity of labelled compound fed).

The physical meaning of relative isotope content is the number of moles of the labelled compound incorporated per mole of the aflatoxins recovered. That is:

$$\text{Relative isotope content} = \frac{A_2/M_2}{A_1/M_1} = \left(\frac{A_2}{A_1/M_1}\right)\Big/M_2 = N_1/M_2 \qquad (1)$$

where A_1 = radioactivity of the labelled compound fed in C; M_1 = quantity of the labelled compound in moles; A_2 = radioactivity of the radioactive aflatoxins recovered in μC; M_2 = quantity of the aflatoxins in μmoles; $(A_2)/(A_1/M_1)$ or N_1 = quantity of the labelled compound, being incorporated into aflatoxins, in μmoles. When the numerator and the denominator of Eqn. 1 are differentiated with respect to time, an instantaneous relative isotope content is obtained as follows.
At any time t,

$$\text{Relative isotope content} = \frac{dN_1}{dM_2} = \frac{dN_1/dt}{dM_2/dt} = \frac{R_1}{R_2} \qquad (2)$$

Where R_1 = rate of incorporation of the labelled compound into aflatoxins in μmoles per h; R_2 = rate of aflatoxin production in μmoles/h.

The relative isotope content so defined can be determined by the ratio of the slope of the incorporation curve to the slope of the production curve at time t.

RESULTS

The composition of aflatoxins produced by this strain of *Aspergillus flavus* under the conditions described is B_1, 78%; B_2, 3%; G_1, 17%; and G_2, 2%. Therefore the concentration of the mixture of aflatoxins could be well approximated by using an extinction coefficient of 20000 as calculated from the appropriate extinction coefficient for individual components[13] and their relative amounts.

As summarized in Fig. 1, the acetate was taken up and incorporated into aflatoxins rapidly, while there was a definite lag period before the radioactive labels from glucose appeared in aflatoxins although the rate of glucose uptake was about the same as that of acetate. Aflatoxins were produced at a constant rate following an initial period of lower rates; the consistency in rate of aflatoxin production occurring in the paired flasks confirms the parallelism. The relative contribution of acetate and glucose as a carbon source for aflatoxin synthesis can be observed by comparing the relative isotope contents for acetate and glucose in the course of incubation. Although glucose and acetate were taken up at about the same rate, aflatoxins were first labelled exclusively by acetate until the exogenous acetate was almost exhausted; and then carbon atoms from glucose started to be incorporated into aflatoxins.

When $^{14}C_6$ glucose was used as a sole carbon source, the rate of glucose uptake was higher and there was no lag period before the incorporation of labels from glucose into aflatoxins, as shown in Fig. 2A. In the presence of acetate however, the lag in incorporation is clearly shown (Fig. 2B).

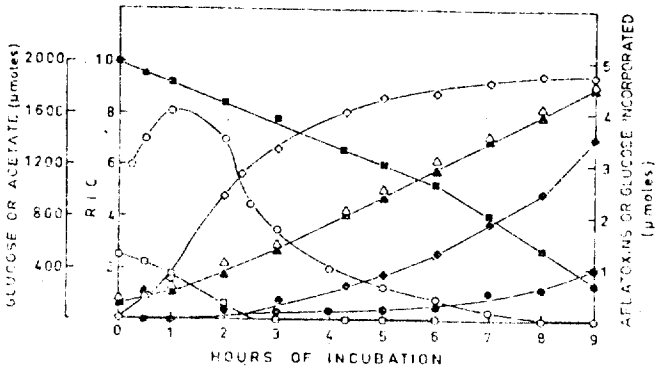


Fig. 1. Simultaneous incorporation of [1-^{14}C] acetate and [$^{14}C_6$] glucose into aflatoxins by parallel cultures contained 100 ml of resting cell medium, 1 g of fresh mycelia, 500 μmoles of acetate, and 2000 μmoles of glucose. In one flask (A) acetate was labelled with [1-^{14}C] acetate (84 μC); in the other flask (B) glucose was labelled with [$^{14}C_6$] glucose (64 μC). Solid data points are obtained from Flask (B), and empty data points from Flask A. □ - □ and ▲ - ▲, aflatoxins; ○ - ○, relative isotope content (RIC) for acetate; ● - ●, relative isotope content (RIC) for glucose; □ - □, residual acetate; ■ - ■, residual glucose; △ - △, acetate incorporated; ◇ - ◇, glucose incorporated.

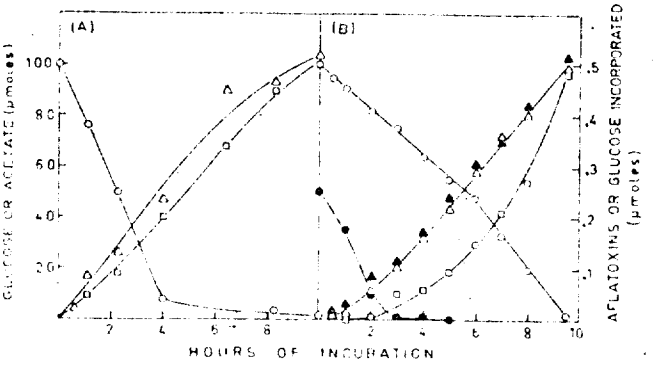


Fig. 2. Incorporation of [$^{14}C_6$] glucose in the absence and presence of acetate during resting cell culture of *Aspergillus flavus*. Each data point represents a separate 10-ml culture agitated at 30° in a 50-ml baffled erlenmeyer flask. The flasks contained 100 μmoles of glucose and where appropriate 50 μmoles of acetate, and 1 g of fresh mycelia. A. Glucose alone: ○ - ○, residual glucose; □ - □, aflatoxins; △ - △, glucose incorporated. B. Glucose and acetate: ○ - ○, residual glucose; ● - ●, residual acetate; □ - □, and ▲ - ▲, aflatoxins; △ - △, glucose incorporated; paired flasks were used as in Fig. 1.

DISCUSSION

The fact that acetate was preferentially incorporated into aflatoxins despite the presence of glucose at a concentration 4 times greater than that of acetate indicates that acetate is a better precursor of aflatoxins than glucose, and that aflatoxins are synthesized by *Aspergillus flavus* in the extramitochondrial soluble fraction of the cells through an acetate-derivative pathway. This result immediately excludes the

involvement of the shikimic acid pathway, which is glucose-derivative, in the synthesis of aflatoxins.

The maximum relative isotope content value obtained using 1-^{14}C-acetate was higher than 8, approaching the hypothetical maximum of 9 as expected from the labelling pattern of aflatoxin-B_1 prepared from the same precursor[3]. The high relative isotope content values provide additional experimental evidence that the ring structure of the aflatoxin is synthesized *via* the polyketide pathway through a head-to-tail assembly of acetate units.

The low relative isotope content obtained on ^{14}C-glucose (Fig. 1) suggests that in the absence of exogenous acetate, aflatoxins are synthesized in large part from intracellular precursor carried over from the growth culture. This was confirmed by the observation that even in the absence of a carbon source the resting cell culture produces small amounts of aflatoxins[11].

The lag in the incorporation of labels from the $^{14}C_6$-glucose into aflatoxins occurring in the presence of acetate, which did not occur in the absence of acetate, indicates that acetate, or a closely related metabolite, controls the formation of the extramitochondrial CoASAc from glucose and that the extramitochondrial CoASAc pool is dominated by the CoASAc derived from exogenous acetate. This regulation may be through the competitive inhibition of pyruvate dehydrogenase which catalyzes the formation of CoASAc from pyruvate as has been found to be the case in rat heart systems[15]. This finding also explains why glucose did not dilute the label from acetate incorporated into aflatoxins[11] during the preparation of highly radioactive aflatoxins from labelled acetate.

The increase in the rate of glucose uptake by the cultures in the absence or after the exhaustion of acetate in the broth shows that acetate, or its metabolite, can inhibit the utilization of glucose by the mold. A similar phenomenon has been observed with *Aspergillus nidulans*[16] and the regulation was also through the exclusion of the sugar from the cells because of the intracellular high level of CoASAc.

ACKNOWLEDGEMENT

This investigation was supported by U.S. Public Health Service Grant EF 00004.

REFERENCES

1 R. I. Mateles and J. C. Adye, *Appl. Microbiol.*, 13 (1965) 208.
2 J. A. Donkersloot, D. P. H. Hsieh and R. I. Mateles, *J. Am. Chem. Soc.*, 90 (1968) 502.
3 M. Biollaz, G. Büchi and G. Milne, *J. Am. Chem. Soc.*, 90 (1968) 5017.
4 C. DeDuve, R. Wattiaux and P. Baudhuin, *Advan. Enzymol.*, 24 (1962) 291.
5 H. R. Mahler and E. H. Cordes, *Biological Chemistry*, Harper and Row, New York and London, 1966, p. 620.
6 D. E. Green and S. Fleischer, in D. M. Greenberg, *Metabolic Pathways*, Vol. 1, Academic Press, New York and London, 1960, p. 41.
7 M. A. Eisenberg, *Biochim. Biophys. Acta*, 16 (1955) 58.
8 P. Berg, *J. Biol. Chem.*, 222 (1956) 991.
9 J. M. Lowenstein, *Symp. Biochem. Soc.*, 24 (1963) 57.
10 J. C. Adye and R. I. Mateles, *Biochim. Biophys. Acta*, 86 (1964) 418.
11 R. M. Epeley, *J. Assoc. Offic. Anal. Chem.*, 49 (1966) 478.
12 L. Stoloff, A. C. Beckwith and M. E. Gershenzon, *J. Assoc. Offic. Anal. Chem.*, 51 (1968) 98.
13 G. N. Wogan, *Bacteriol. Rev.*, 30 (1966) 795.
14 D. P. H. Hsieh, Sc. D. Thesis, M.I.T., Cambridge, Mass., 1969, p. 91.
15 P. B. Garland and P. J. Randle, *Biochem. J.*, 91 (1964) 6c.
16 A. H. Romano and H. L. Kornberg, *Biochim. Biophys. Acta*, 158 (1968) 501.

Brit. J. Haemat., 1968, 15, 203

Studies on the Incorporation of ^{14}C-Sodium Acetate into the Phospholipids of Phytohaemagglutinin-Stimulated and Unstimulated Lymphocytes

H. Huber,* N. Strieder, H. Winnler,† G. Reiser and K. Koppelstaetter

Medizinische Klinik und Pharmakolgisches Institut der Universitaet, Innsbruck, Austria

Summary. The *in vitro* incorporation of ^{14}C-acetate into the phospholipids of unstimulated and phytohaemagglutinin (PHA)-stimulated lymphocytes and the distribution of radioactivity in the phospholipid fractions, separated by thin-layer chromatography, have been studied in lymphocyte cultures.

The percentage of radioactive incorporation into the phospholipid fractions of PHA-stimulated lymphocytes was as follows: lysolecithin 4.0 (± 1.3), sphingomyelin 12.7 (± 1.4), lecithin 39.2 (± 2.0), phosphatidyl-serine and phosphatidyl-inositol 14.1 (± 2.1), phosphatidyl-ethanolamine 28.5 (± 2.0). The percentage of radioactive incorporation into the phospholipid fractions of unstimulated lymphocytes was: lysolecithin 7.9 (± 1.4), sphingomyelin 8.2 (± 1.4), lecithin 34.5 (± 2.6), phosphatidyl-serine and phosphatidyl-inositol 14.5 (± 1.3), phosphatidyl-ethanolamine 31.4 (± 1.4).

The results of the lipid-phosphorus distribution ($n = 4$) were similar and showed differences only in the sphingomyelin fraction ($P < 0.05$). With a given initial cell number, the radioactive incorporation into the phospholipid fractions of PHA-stimulated lymphocytes was increased by the following factors: lysolecithin 3.9 (± 0.6), sphingomyelin 7.0 (± 1.5), lecithin 8.1 (± 1.5), phosphatidyl-serine and phosphatidyl-inositol 8.8 (± 1.3), phosphatidyl-ethanolamine 7.3 (± 0.8).

Cell-fractionation experiments with PHA-stimulated lymphocytes after labelling with ^{3}H-acetate showed a similar distribution of the radioactivity in the phospholipids of 'mitochondria' and 'microsomes'.

Up to now only a few results have been reported on the phospholipid synthesis of lymphoid cells. Such experiments have been carried out on cells of patients with leukaemia (Lukin and Williams, 1961), on cells of the lymphatic duct (Blomstrand, 1966) and on thymus lymphocytes (Rose and Frenster, 1965).

The transformation of small lymphocytes after addition of phytohaemagglutinin (PHA) leads to an increased metabolic activity and to the appearance of lipid-containing vacuoles (Gropp and Fischer, 1966; Fischer and Gropp, 1966). The changes in phospholipid synthesis in PHA-stimulated lymphocytes, however, have not been evaluated.

In the present paper we are reporting on (a) the incorporation of labelled sodium acetate

* Present address: San Francisco Medical Center, Section of Hematology and Immunology, California, U.S.A.
† Present address: University Department of Pharmacology, Oxford

into the phospholipid fractions of largely 'pure' lymphocyte suspensions, and (b) the changes in the phospholipid synthesis after stimulation by PHA.

MATERIALS AND METHODS

Blood samples (80 ml.) were taken from healthy adults into heparinized and sterilized screw-cap tubes (Heparin free of cresol 50 I.U./ml. blood). Plasma rich in leucocytes was obtained by spontaneous sedimentation. Lymphocytes were separated by the method of Rabinowitz (1964), using a column filled with glass beads as described in detail by Cinesa and Huber (1968). The percentage of lymphocytes of the eluted leucocytes amounted to 98 ± 0.9 per cent ($n = 22$); the number of erythrocytes varied between one and four erythrocytes per lymphocyte. Under the conditions of these experiments thrombocytes were not separated from the plasma. The cells were cultured in medium TC 199 and in homologous plasma (final concentration 33 per cent v./v.). At the beginning of the experiments the number of cells was 5×10^5 lymphocytes/ml. culture. PHA (Wellcome) in a final concentration of 0.05 ml./ml. incubate was added to half of the cultures. About 54 hours after the start 1 μCi ^{14}C-sodium-acetate per ml. incubate (specific acticity 28.2 mCi/mM, Radiochemical Centre, Amersham) was added. Labelling was stopped after 6 hours. The cells were sedimented by centrifugation at 350 g for 10 minutes, erythrocytes were haemolysed using 0.9 per cent NH_4Cl, and the sediment was washed three times in normal saline. The cells were then dispersed in 0.5 ml. distilled water and diluted with a 20-fold volume of chloroform–methanol (2 : 1, v./v.) in order to extract the lipids. This extract was washed four times according to Folch, Lees and Sloane-Stanley (1957), dried under a stream of nitrogen and dissolved in a small volume (0.02–0.04 ml.) of chloroform–methanol.

Separation of the phospholipids was carried out by thin-layer chromatography on 20 × 20 plates (Silica-Gel 'H' Merck, layer-thickness 0.5 mm.). Chromatograms were developed with chloroform–methanol–acetic acid–water 75 : 45 : 12 : 6, v./v.) as described by Skipski, Peterson and Barclay (1964). For quantitative determination the phospholipid spots were made visible by means of iodine vapour and their positions were marked. As a carrier lipid, rat liver extract was added to the extract of lymphocytes. When most of the iodine had been evaporated the individual spots were scraped into scintillation tubes. Radioactivity was read in a liquid-scintillation counter (Packard Instruments, Inc.). To confirm the identity of the spots the following reagents were employed: (a) ninhydrin for phospholipids containing free-amino groups, (b) the Dragendorff-reagent for those containing choline, and (c) reference phospholipids. The following individual spots were identified: lysolecithin, sphingomyelin, lecithin, phosphatidyl-ethanolamine. Since it was not possible to separate phosphatidyl-serine and phosphatidyl-inositol these two phospholipids were determined together. The lipid phosphorus of the extract of lymphocytes without carrier lipids was determined by the method of Bartlett (1959).

Preliminary experiments had shown that thrombocytes, after cultivation under the same conditions as the lymphocytes, do not show any incorporation of the acetate into the phospholipids. The thrombocytes were harvested by differential centrifugation of the plasma rich in leucocytes (Huber, Huber and Platzer, 1966).

PHA-stimulated lymphocytes were labelled with 10 μCi ^{3}H-sodium-acetate (specific

activity 500 mCi/mM, Radiochemical Centre, Amersham) per ml. culture (6×10^6 cells were cultivated) and subjected to differential centrifugation as described earlier (Huber, Winkler, Reiser, Huber, Gabl and Braunsteiner, 1967). The phospholipids of Sediment II ('mitochondria') and sediment III ('microsomes') were separated as described. Since sediment I was contaminated with unbroken cells it was not evaluated.

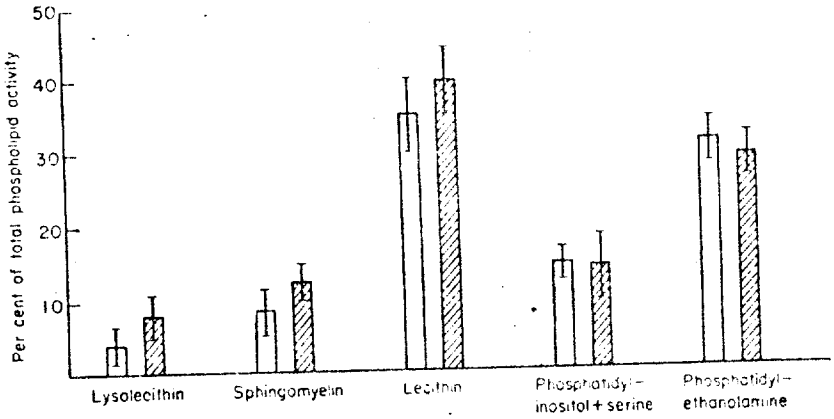


FIG. 1. Incorporation of ^{14}C-acetate into the phospholipids of PHA-stimulated (shaded columns) and unstimulated (open columns) lymphocytes. (Five experiments.)

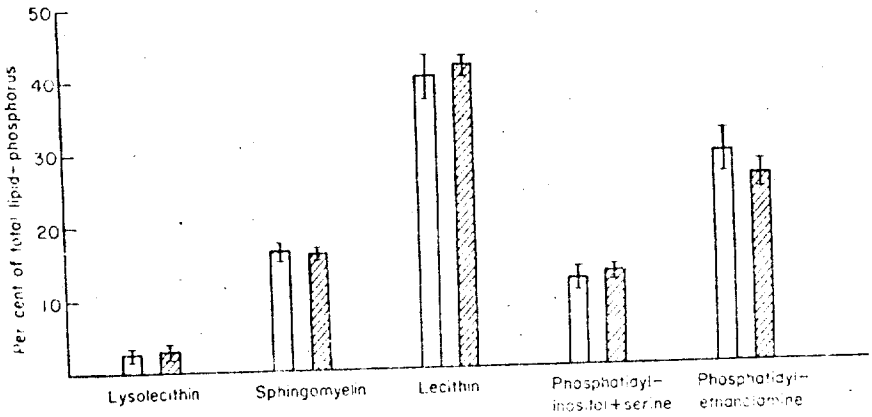


FIG. 2. Relative amounts of lipid phosphorus in the different phospholipid fractions of PHA-stimulated (shaded columns) and unstimulated (open columns) lymphocytes. (Four experiments.)

RESULTS

Fractions of Phospholipids in Unstimulated and PHA-Stimulated Lymphocytes

The results of ^{14}C-acetate incorporation in PHA-stimulated and unstimulated lymphocytes are shown in Fig. 1. About one-third of the radioactivity was found in the fraction of

lecithin and of phosphatidyl ethanolamine. The lowest activity was found in lysolecithin whereas intermediate values were obtained for phosphatidyl-inositol, phosphatidyl-serine and sphingomyelin. The percentage of incorporated activity was similar for peripheral lymphocytes stimulated with PHA and for unstimulated ones.

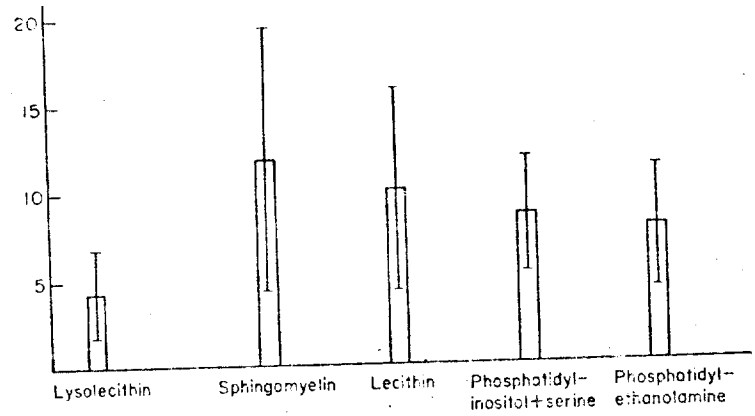


FIG. 3. Increase of ^{14}C-acetate incorporation into phospholipids after stimulation by PHA. Values are expressed as ratio of ^{14}C radioactivity in phospholipids of stimulated to unstimulated cultures. (Five experiments.)

TABLE I

RELATIVE AMOUNTS OF LIPID PHOSPHORUS IN THE DIFFERENT PHOSPHOLIPID FRACTIONS OF PHA-STIMULATED AND UNSTIMULATED LYMPHOCYTES: COMPARISON WITH RELEVANT DATA

	No. of experiments	*Lysolecithin*	*Sphingomyelin*	*Lecithin*	*Phosphatidyl-inositol*	*Phosphatidyl-serine*	*Phosphatidyl-ethanolamine*	*Reference*
Lymphocytes cultured without PHA	4	2.5 ± 0.9	16.5 ± 1.2	39.9 ± 3.2	12.2 ± 1.3		28.8 ± 5.7	Present study
Lymphocytes cultured with PHA	4	3.0 ± 1.1	16.1 ± 0.8	41.3 ± 1.2	12.9 ± 1.1		25.6 ± 1.9	Present study
Thymus-lymphocytes	3		5.8	57.2	0.3	0.2	34.2	Rose and Frenster (1965)
Guinea-pig leucocytes	14		18 ± 4	35 ± 5	4 ± 2	4 ± 1	35 ± 5	Kornovski and Wallach (1961)
Leucocytes of patients with chronic myeloid leukaemia	5		11	44	6	9	29	Firkin and Williams (1961)

The results of the lipid phosphorus determination in unstimulated and in PHA-stimulated lymphocytes are shown in Fig. 2 and Table I. The results obtained by the two methods were comparable. The relative amounts of ^{14}C-acetate incorporation and of the lipid phosphorus distribution differed significantly only in the phospholipid fraction ($P<0.05$).

Since residues of erythrocytes had been left in the sediment the specific activity was not determined.

Increased Incorporation of ^{14}C-Acetate into the Phospholipids of PHA-Stimulated Lymphocytes

The summarized data in Fig. 3 show that the increase of ^{14}C-acetate incorporation was comparable in all phospholipid fractions except in lysolecithin. With an equal number of cells at the start, the total incorporation into the phospholipids of PHA-stimulated lymphocytes was 7.5 ($\pm$ 1.2) times higher than in unstimulated cells.

Subcellular Distribution of Phospholipids in Stimulated Lymphocytes

Fig. 4 gives the results of three cell-fractionating experiments. It shows that the distribution of radioactivity was quite similar in the phospholipids of sediment II ('mitochondria')

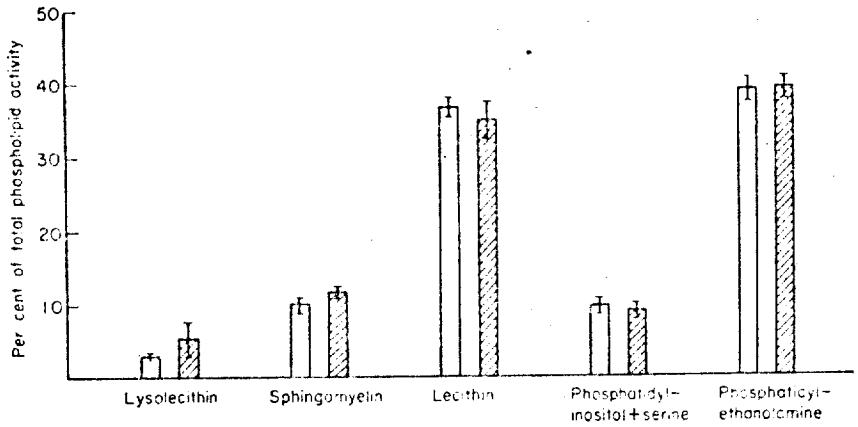


FIG. 4. Incorporation of ^{14}C-acetate into phospholipid fractions of mitochondia (open columns) and microsomes (shaded columns). (Three experiments.)

and of sediment III ('microsomes'). Evaluation of the cell sap was not performed as the radioactivity was too low.

DISCUSSION

The present *in vitro* investigations of the incorporation of ^{14}C-acetate into the phospholipids of peripheral lymphocytes show that the highest percentage of incorporation took place in lecithin, phosphatidyl-ethanolamine and sphingomyelin, the lowest in lysolecithin. Similar results were obtained with PHA-stimulated lymphocytes. Our cultures were largely free from granulocytes and monocytes; contaminating erythrocytes were haemolysed. Mature erythrocytes show, in contrast to intact thrombocytes, no phospholipid synthesis (Buchanan, 1960; Marks, Gellhorn and Kidson, 1960). Preliminary investigations had shown that no ^{14}C-acetate incorporation into the phospholipids of thrombocytes occurred under our experimental conditions. It seems that the observed incorporation is due to a metabolic activity of the cultivated lymphocytes.

Our findings agree with the results of other authors on lymphocytes and other white

cells. Such investigations have been made on leucocytes of patients with leukaemia (Finlin and Williams, 1961; Malamos, Miras, Levis and Mantzos, 1962), on cells of the lymphatic duct (Blomstrand, 1966), on thymus lymphocytes (Rose and Frenster, 1965) and on granulocytes of guinea-pigs (Karnovsky and Wallach, 1961). The results are summarized in Table I. In spite of differences in the methods, these results are comparable with our findings on isolated lymphocytes, indicating a similarity in the distribution of phospholipids in various white blood cells, in thymus lymphocytes and in lymphocytes transformed by PHA-stimulation. The percentage distributions of the phospholipids determined by lipid-phosphorus assay and ^{14}C-acetate incorporation were similar in our cultures of lymphocytes except in the case of sphingomyelin, where the two methods of determination show a difference, with lower figures from the second method. Our results agree with the findings of other authors that the turnover of sphingomyelin was slow compared with that of other phospholipids (Paysant-Diament and Polonovski, 1960).

Mitochondria and microsomes play a leading part in the synthesis of phospholids (Ansell and Hawthorne, 1964). Our results from cell fractionation experiments indicate that the labelling of phospholipids in these two subcellular fractions is similar; however, cardiolipin was not separated. A similar phospholipid composition, with the exception of cardiolipin, has been found in different organelles of various tissues (Rose and Frenster, 1965; Ansell and Hawthorne, 1964; MacFarlane, Gray and Wheeldon, 1960).

Only a small percentage of ^{14}C-acetate was incorporated into the lysolecithin fraction, and only a slight increase was found after stimulation with PHA. Lysolecithin is a membrane-active compound which may play a role in immunological cytolysis (Fischer and Haupt 1961). However, despite the cytotoxic effect of PHA-stimulated lymphocytes (Hohn, Permann and Werner, 1964), we found no indication of an increased lysolecithin synthesis in these cells. Nor was there a preferred synthesis of phosphatidyl-inositol which is produced along with phosphatidic acid in various secreting cells (Eggman and Hokin, 1960) and in granulocytes during phagocytosis (Karnovsky and Wallach, 1961).

In comparison to cultures of 'resting' lymphocytes, those with added PHA incorporated on the average more than seven times as much ^{14}C-acetate into the phospholipids. Our results suggest a quantitative increase in phospholipid synthesis rather than a qualitative change. The findings are comparable, therefore, with those on protein synthesis (Huber *et al.*, 1967) where no single protein is formed preferentially, but many of the proteins present in 'resting' lymphocytes are increased.

ACKNOWLEDGMENT

This work was supported from the funds 'Spendenaktion Kampf dem Krebs'.

REFERENCES

ANSELL, G. B. and HAWTHORNE, J. N. (1964). *Phospholipids*. Elsevier, Amsterdam.

BARTLETT, G. R. (1959). 'Phosphorus assay in column chromatography.' *J. Biol. Chem.*, **234**, 466.

BLOMSTRAND, R. (1966). 'Fatty acid synthesis in human lymphocytes.' *Acta chem. scand.*, **20**, 1122.

BUCHANAN, A. A. (1960). 'Lipid synthesis by human leucocytes *in vitro*.' *Biochem. J.*, **75**, 315.

CIRESA, M. and HUBER, H. (1968). 'Die Isolierung funktionsfähiger Blutlymphozyten.' *Acta haemat. (Basel)*. (In press).

EUGMAN, L. D. and HORIN, L. E. (1960). 'The relationship between secretory activity and the incorporation of P^{32} into phosphoinositide and phosphatidic acid in salivary glands and pigeon esophageal mucosa *in vitro*.' *J. biol. Chem.*, **235**, 2869.

FIRKIN, B. G. and WILLIAMS, W. J. (1961). 'The incorporation of radioactive phosphorus into the phospholipids of human leukemic leukocytes and platelets.' *J. clin. Invest.*, **40**, 423.

FISCHER, H. and HAUPT, I. (1961). 'Das cytolysierende Prinzip von Serumkomplement.' *Z. Naturf.*, **16b**, 321.

FISCHER, R. and GROPP, A. (1966). 'Cytochemie der Lymphocyten in vitro.' *Klin. Wschr.*, **44**, 733.

FOLCH, J., LEES, M. and SLOANE-STANLEY, G. H. (1957). 'A simple method for the isolation and purification of total lipides from animal tissues.' *J. biol. Chem.*, **226**, 497.

GROPP, A. and FISCHER, R. (1966). 'Ergebnisse der Züchtung von Lymphocyten *in vitro*.' *Klin. Wschr.*, **44**, 695.

HOLM, G., PERLMANN, P. and WIGZELL, B. (1964). 'Phytohaemagglutinin-induced cytotoxic action of normal lymphoid cells on cells in tissue culture.' *Nature (Lond.)*, **203**, 841.

HUBER, H., HUBER, V. and PLATZER, S. (1966). 'Untersuchungen zur Thrombozytenlebensdauer bei Leberzirrhose mit $DF^{32}P$.' *Wien. klin. Wschr.*, **78**, 869.

HUBER, H., WINKLER, H., REISER, G., HUBER, C., GABL, F. and BRAUNSTEINER, H. (1967). 'Eigenschaften und subcelluläre Lokalisation neugebildeter Proteine menschlicher Lymphozyten in vitro, mit und ohne Phytohämagglutininzusatz.' *Klin. Wschr.*, **45**, 204.

KARNOVSKY, M. L. and WALLACH, D. F. H. (1961). 'The metabolic basis of phagocytosis. III. Incorporation of inorganic phosphate into various classes of phosphatides during phagocytosis.' *J. biol. Chem.*, **236**, 1895.

MACFARLANE, M. G., GRAY, G. M. and WHEELDON, L. W. (1960). 'Fatty acid composition of phospholipids from subcellular particles of rat liver.' *Biochem. J.*, **77**, 626.

MALAMOS, B., MIRAS, C., LEVIS, G. and MANIZON, J. (1962). 'The *in vitro* incorporation of acetate-1-C^{14} into normal and leukemic leukocyte lipids.' *J. Lipid Res.*, **3**, 222.

MARKS, P. A., GELLHORN, A. and KIDSON, C. (1960). 'Lipid synthesis in human leukocytes, platelets and erythrocytes.' *J. biol. Chem.*, **235**, 2579.

PAYSANT-DIAMENT, M. and POLONOVSKI, J. (1960). 'Incorporation *in vitro* du ^{32}P dans les phospholipides globulaires du sang humain.' *Bull. Soc. Fr.*, **42**, 337.

RABINOWITZ, Y. (1964). 'Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns, including tissue culture observations.' *Blood*, **23**, 811.

ROSE, H. G. and FRENSTEP, J. H. (1965). 'Composition and metabolism of lipids within repressed and active chromatin of interphase lymphocytes.' *Biochim. biophys. Acta (Amst.)*, **106**, 577.

SKIPSKI, V. P., PETERSON, R. F. and BARCLAY, M. (1964). 'Quantitative analysis of phospholipids by thin-layer chromatography.' *Biochem. J.*, **90**, 374.

WAGNER, H., HÖRHAMMER, L. and WOLFF, P. (1961). 'Dünnschichtchromatographie von Phosphatiden und Glykolipiden.' *Biochem. Z.*, **334**, 175.

Formation of Monounsaturated Fatty Acids in Fatty Liver

J. Biochem. (Tokyo) 61(6): 712-18, (1967)

By Yoh Imai and Osamu Mitsuhashi

(*From the Department of Biochemistry, Hokkaido University School of Medicine, Sapporo*)

(Received for publication, August 2, 1966)

1. Monoenoic acid formation from stearic acid was studied with liver preparations from starved and CCl_4-intoxicated rats. The rate of monounsaturation decreased both in starved and CCl_4-treated animals, while the oleic acid content increased in preparations of these fatty livers. Freeze-thawing of liver preparations reduced the desaturating activity.

2. The ^{14}C-distribution pattern in oleic acid molecules derived from 1-^{14}C-stearic acid and from 1-^{14}C-acetic acid, suggests that an intact 18-carbon unit is converted directly to oleic acid even in fatty liver, and that acetic acid may serve as a precursor of oleic acid only *via* stearic acid.

Since Imai first showed that desaturation of saturated fatty acids decreases in diabetic (*1*) and fasting animals (*2*), several workers reported a distinct depression of monoenoic fatty acid synthesis in fat infiltrated livers (*3*) and in adipose tissues of diabetic rats (*4*).

It is generally observed, however, that the concentration of monounsaturated fatty acid increases in fatty livers (*5, 6*). If both observations are true, the mechanism of formation of monoenoic acids should differ in normal and fatty livers.

With regard to the biosynthesis of monoenoic fatty acids, Bloomfield and Bloch reported a possible mechanism for the direct desaturation of long chain fatty acid in yeast (*7*). By this mechanism, only the carboxyl carbon of the monoenoic acid molecule should be labeled, when the corresponding 1-^{14}C-saturated fatty acid is used as precursor.

Another possible mechanism for the synthesis of monoenoic acids, described by Scheuerbrandt and Bloch for anaerobic microorganisms (*8*), produces olefinic double bonds during the process of chain-lengthening. If this reaction occurs in fatty liver, a 1-^{14}C-saturated long chain acid should first be split into labeled C_2-units and unlabeled short chain fatty acid. Then the labeled atoms should mainly be incorporated into the carboxyl side of the newly synthesized olefinic molecules.

A third possibility was reported by Barron and Stumpf (*9*), who suggested the olefinic acids in higher plants might be synthesized from C_2-units by a different pathway from saturated fatty acids. If this occurs in fatty liver, even with a long chain 1-^{14}C-saturated fatty acid as precursor, the acid should first be split into labeled C_2-units. Then a monoenoic acid is resynthesized from the labeled C_2-units. Thus labeled carbons would be evenly distributed in the resulting monoenoic acid molecule.

Thus several possible mechanisms have been suggested for the biosynthesis of monoenoic acids. Each would result in a different distribution of radioactivity in the product (Fig. 1). Therefore, using 1-^{14}C-saturated fatty acid as a precursor, it should be possible to deduce the mechanism of desaturation from the distribution of labeled atoms in the biosynthetic olefinic acid.

In the present study, the labeling of biosynthetic oleic acid in normal and fatty livers

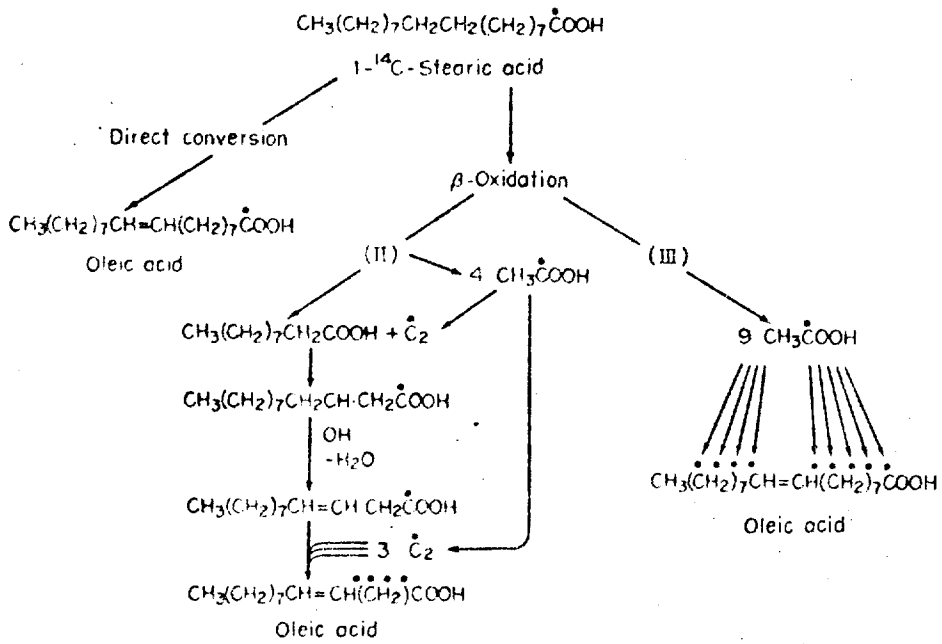


FIG. 1. Possible mechanisms of oleic acid biosynthesis from 1-^{14}C-stearic acid.

was examined using 1-^{14}C-stearic acid and 1-^{14}C-acetic acid as substrates.

EXPERIMENTAL AND METHODS

Animals. Male Wistar strain rats, weighing 150 g. were used. They were maintained on synthetic rat chow* *ad libitum*. Starved animals were fasted for 24 or 48 hours. In CCl_4-ingestion experiments, rats were given olive oil (0.25 ml. per 100 g. body weight) or a CCl_4-olive oil (1 : 1) mixture (0.5 ml. per 100 g. body weight) by stomach tube, and 4 hours later the livers were removed.

Tissue Preparation and Incubation Procedure. Livers were homogenized in 2.5 volumes of potassium phosphate buffer (pH 7.4) containing nicotinamide, as described by Clayton and Bloch (*10*), and the homogenates were centrifuged at 10,000×*g* for 20 minutes. The supernatant fraction was used for incubation, either immediately, or after heating, or after freeze-thawing three times (4 minutes freezing and 3 minutes thawing of the supernatant, designated as the "aged supernatant" (*11*).

The incubation mixture contained 0.8 ml. of fresh (or aged) supernatant, 0.2 ml. of 0.5 *M* potassium phosphate buffer (pH 7.4) and 0.03 ml. of 1-^{14}C-stearic acid* (100 mμmoles) emulsion in Tween 60, in a total volume of 1 ml. Incubation was carried out for 1 hour at 37 C with shaking.

Extraction and Separation of Fatty Acids.—Incubation was terminated by the addition of 1 ml. of 50 per cent KOH solution and 0.5 ml. of methanol. Then the mixture was heated for 30 minutes at 85°C, cooled, and unsaponified lipids were removed with petroleum ether. The remaining mixture was acidified, and fatty acids were extracted with ether. The ethereal layer was washed with water and then dried on anhydrous sodium sulfate.

The non-polar acids, including the saturated substrate and the monoenoic product, were first freed from polar acids according to the method of Preiss and Bloch (*11*) as follows: The fatty acid mixture, dissolved in 5 ml. of ether-pentane (15 : 85, v/v), was applied on a silicic acid (Mallinkrodt, 600–700 mesh) column. The eluate, containing the non-polar acids (90% recovery), was esterified with diazomethane. The esters were then separated into saturated and

* Radioactive 1-^{14}C-stearic acid, purchased from Daiichi Kagaku Corporation, was purified by chromatography on a silicic acid column as described by Preiss and Bloch (*11*).

monoenoic esters by silver nitrate-silica gel TLC* with petroleum ether (30–60° b.p.)-ether (90:7) mixture. The R_f values were 0.6–0.7 for saturated esters, 0.3–0.5 for monoenoic esters and 0.2–0.3 for dienoic esters. The saturated and monoenoic acid esters were extracted separately with ether and purified by chromatography on a small silicic acid (0.3–0.5 g.) column. The eluate was collected in weighed planchets. The radioactivities of the two samples were determined directly with a gas-flow counter, to test the rate of monoenoic acid formation.

Degradation of Labeled Fatty Acids—After addition of 50 mg. of carrier oleic acid, the monoenoic acids were oxidized by the permanganate-periodate procedure as described by Bloomfield and Bloch (*7*). The resulting short-chain fatty acids were extracted thoroughly from the reaction mixture with ether. Then they were separated into monocarboxylic acids (representing the methyl side of the original olefinic acid) and dicarboxylic acids (representing the carboxylic side of the original olefinic acid), by thin layer chromatography on silicic acid (Millincrodt) with ether-pentane (15:85, v/v) as solvent. Each fraction was extracted with ether for determination of radioactivity.

The decarboxylation of long chain fatty acid was carried out *via* the Schmidt reaction with HN_3, according to the microprocedure of Brady *et al.* (*12*), in a small Warburg type flask. The $^{14}CO_2$ liberated was trapped in saturated KOH solution in the center-well of the flask, and converted to $Ba^{14}CO_3$ by the addition of half saturated $Ba(OH)_2$ solution containing 2% $BaCl_2$ (*13*). The recovery of isotopic carbon was 95 per cent or more either from standard 1-^{14}C-stearic acid or from purified 1-^{14}C-oleic acid.

RESULTS AND DISCUSSION

Rate of Monoenoic Acid Formation—Liver preparations were incubated with 1-^{14}C-stearic acid at 37°C for a specified period. Then fatty acids were extracted from the reaction mixture and fractionated into saturated and monoenoic acids. The rate of formation of monoenoic acid was calculated as:

$$\frac{\text{Radioactivity in monoenoic acid}}{\text{Radioactivity in saturated and monoenoic acids}}$$

The liver preparations from well fed rats yielded 3.2 and 7.3% monoenoic acid in 30 and 60 minutes, with 1-^{14}C-stearic acid as substrate (Table I). In subsequent experiments, incubation was performed for 60 minutes to minimize isotopic randomization in the monoenoic acid molecule.

As shown in Table II, the rate of monounsaturation of stearic acid decreased in starved animals. Prolonged starvation further reduced the desaturation of stearic acid. Freeze-thawing of the liver preparations also reduced the desaturation process. Therefore, the enzymes related to the monounsaturation of stearic acid seem to be extremely unstable.

Histologically, the livers of CCl_4-intoxicated rats showed fatty infiltration. The fatty acid composition of the lipids of the 10,000×g supernatant fraction of the livers was analyzed. As shown in Table III, the oleic acid content was markedly increased in liver preparations from CCl_4-intoxicated rats as compared with those from control animals. Desaturation of 1-^{14}C-stearic acid was very low even in fresh preparations from CCl_4-intoxicated rats (Table II).

Mechanism of Monounsaturation—In a preliminary experiment, using 1-^{14}C-stearic acid as a precursor, isotopically labeled monoenoic acids seemed to be mainly oleic acid. The monoenoic acid fraction produced was esterified with diazomethane and subjected to gas chromatography with a thermal conductivity detector. Then the eluted gas was subjected to fractional trapping in a cotton column immersed in cold methanol. Most of the radioactivity was detected in the oleic acid fraction. This suggests that oleic acid was directly formed from stearic acid.

To investigate the mechanism of monoenoic acid formation, liver preparations were incubated with 1-^{14}C-stearic acid or 1-^{14}C-acetic acid, and the resulting oleic acid was isolated and the distribution of ^{14}C in the oleic acid molecule was determined.

As shown in Tables IV and V, the ^{14}C-distribution in the oleic acid molecules, produced by liver preparations from normal and CCl_4-intoxicated rats, were almost the same. For instance, with 1-^{14}C-stearic acid as precursor (Table IV), three quarters of the radioactivity in the resulting oleic acid molecule was in the carboxylic side, in which most of the radioisotopes were localized in a carboxyl-carbon. Therefore, desaturation mechanisms II and III

* 30 g. of Kiesel Gel G (Merck) were mixed with 65 ml. of 5 per cent $AgNO_3$ solution.

of Fig. 1 are eliminated. Thus, from the results, the intact 18-carbon unit of stearic acid seems to be converted to oleic acid.

^{14}C-Oleic acid, derived from 1-^{14}C-acetic acid, which is known to label the odd numbered carbons of stearic acid, also showed the expected pattern in both normal and CCl_4-intoxicated rats. The radioactivity was evenly distributed in the biosynthetic oleic acids (Table V).

TABLE I

Oleic Acid Formation from Stearic Acid in Liver Preparation of Normal Rats

Incubation period	Fatty acid isolated: As stearic acid (a)	Fatty acid isolated: As oleic acid (b)	Rate of conversion (c)
min.	c.p.m.	c.p.m.	%
0	3,466	56	1.6
30	2,900	77	3.2
60	2,394	142	7.3
120	3,010	480	16.4
(boiled enzyme)			
60	8,181	48	0.6

(c) Calculated as $100\times(b)/(a+b)$.

Conditions of experiments given in the text. Each flask contained 25 mg. of protein.

TABLE II

Reduced Rates of Desaturation in Liver Preparations from Starved and CCl_4-intoxicated Rats

Conditions of animal	Number of rats used	Protein per flask (mg.)	Rate of desaturation[1]: Fresh perparation (%)	Rate of desaturation[1]: Aged preparation[2] (%)
Normal	2	24.8–29.8	6.45±1.67	2.10±1.20
Starved 24hr.	2	18.6–31.0	3.05±1.33	1.00±0.70
Starved 48hr.	2	28.6–31.3	1.05±1.45	0.60±0.35
CCl_4-intoxicated	4	21.5–30.0	0.38±0.26	0.55±0.35

Incubation for 60 minutes.

1) Calculated as described for Table I. Values are given as mean±standard error calculated from the results obtained in 4 or more experiments.

2) After 4 minutes freezing and 3 minutes thawing the supernatant three times.

TABLE III

Composition of Fatty Acids from Liver Preparations (10,000×g supernatant)

Rat group	Fatty acid %							
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Control	0.4	26.0	3.2	15.7	20.0	20.0	2.3	11.0
CCl_4-intoxicated	0.8	27:7	5.3	7.1	25.8	24.9	2.9	5.6

TABLE IV

^{14}C-Distribution in Oleic Acid Molecule Derived from 1-^{14}C-Stearic Acid

Liver preparations from	Total activity[1] (a)	Monocarboxylic acids: Activity (b)	Monocarboxylic acids: Radio-distribution (c)	Dicarboxylic acids: Activity (d)	Dicarboxylic acids: Radio-distribution (e)	Carboxylic carbon: Activity (f)	Carboxylic carbon: Radio-distribution (g)
	c.p.m.	c.p.m.	(%)	c.p.m.	(%)	c.p.m.	%
Control rats	4,860	—	—	—	—	2,800	59
	4,500	840	24	2,700	76	—	—
				$\underbrace{CH_3(CH_2)_7CH}_{24}=\overbrace{CH(CH_2)_7\underbrace{COOH}_{59}}^{76}$			
CCl_4-intoxicated rats	2,720	—	—	—	—	1,760	64
	4,200	727	24	2,260	76	—	—
				$\underbrace{CH_3(CH_2)_7CH}_{24}=\overbrace{CH(CH_2)_7\underbrace{COOH}_{64}}^{76}$			

(b) Calculated as 100×(b)/(b+d) (e) Calculated as 100×(d)/(b+d) (g) Calculated as 100×(f)/(a)

1) ^{14}C-oleic acid collected from 10 or more samples.

TABLE V

^{14}C-Distribution in Long-chain Saturated Acids and Oleic Acid Derived from 1-^{14}C-Acetic Acid

Liver preparation from	Saturated acid[1]				Oleic acid[2]							
		Carboxylic-carbon			Monocarboxylic acid		Dicarboxylic acid		Carboxylic carbon	
	Total activity (a)	Activity (b)	Radio-distribution (c)	Total activity (d)	Activity (e)	Radio-distribution (f)	Activity (g)	Radio-distribution (h)	Activity (i)	Radio-distribution (j)
	c.p.m.	c.p.m.	%	c.p.m.	c.p.m.	%	c.p.m.	%	c.p.m.	%
Control rats	3,025	575	19	2,113	636	43	858	57	—	—
	—	—	—	2,600	—	—	—	—	410	16
	$CH_3(CH_2)_nCOOH$ 81				$CH_3(CH_2)_7CH-CH(CH_2)_7COOH$ 43			57		
										16
CCl_4-intoxicated rats	3,400	720	21	3,270	1,020	41	1,480	59	—	—
	—	—	—	4,380	—	—	—	—	1,050	24
	$CH_3(CH_2)_nCOOH$ 79		21		$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$ 41			59		
										24

(f) Calculated as 100×(e)/(e+g) (h) Calculated as 100×(g)/(e+g)

1: Collected from 2 or more samples 2: Collected from 10 or more samples

Thus the patterns of labeling of the oleic acid molecule differed with the two different ^{14}C-precursors, stearic acid and acetic acid. When ^{14}C-acetate is used as precursor, stearic acid is probably formed first, and is then converted directly to oleic acid, both in normal and CCl_4 intoxicated rats.

The high oleic acid content of the livers of CCl_4-intoxicated rats may be more closely related to disorders of transportation or catabolism of the substance than to its biosynthesis, because oleic acid formation from both stearic acid and acetic acid is greatly diminished in fatty liver (*1, 3*).

The authors wish to express their deep gratitude to Professor Emeritus M. Yasuda, under whose direction an early part of this work was carried out.

REFERENCES

(*1*) Imai, Y., *J. Biochem.*, **49**, 642 (1961)
(*2*) Imai, Y., *J. Japan. Biochem. Soc.* (in Japanese), **34**, 428 (1962); **35**, 556 (1963)
(*3*) Uchiyama, M., Nakagawa, S., and Okui, S., "Lipid Biochem. Research" (in Japanese), Japan Congr. Biochem. Lipids, Tokyo, p. 33 (1965)
(*4*) Benjamin, W., and Gellhorn, A., *J. Biol. Chem.*, **239**, 64 (1964)
(*5*) Evans, J.D., Oleksyshyn, N., Luddy, F.E., Barford, R.A., and Riemenshneider, R.W., *Arch. Biochem. Biophys.*, **85**, 317 (1959)
(*6*) Swell, L., Law, M.D., and Treadwell, C.R., *J. Nutrition*, **76**, 429 (1962)
(*7*) Bloomfield, D.K., and Bloch, K., *J. Biol. Chem.*, **235**, 337 (1960)
(*8*) Scheuerbrandt, G., and Bloch, K., *J. Biol. Chem.*, **237**, 2064 (1962)
(*9*) Barron, E.J., and Stumpf, R.K., *J. Biol. Chem.*, **237**, PC613 (1962)
(*10*) Clayton, R.B., and Bloch, K., *J. Biol. Chem.*, **218**, 305 (1956)
(*11*) Preiss, B., and Bloch, K., *J. Biol. Chem.*, **239**, 85 (1964)
(*12*) Brady, R.O., Bradley, R.M., and Trams, E.G., *J. Biol. Chem.*, **235**, 3093 (1960)
(*13*) Imai, Y., *J. Biochem.*, **53**, 50 (1963)

STUDIES ON THE PREPARATION OF FISH PROTEIN CONCENTRATE

Fishery Tech. 5 (2) 53-59, (1963)

P. K. ISMAIL, P. MADHAVAN AND V. K. PILLAI
Central Institute of Fisheries Technology, Ernakulam

The paper deals with the method of preparation of an edible fish protein concentrate from cheap miscellaneous fish. The method consists in cooking the fish with 0.5% glacial acetic acid, and extracting batch-wise, using ethyl alcohol followed by an azeotropic mixture of hexane and alcohol (B. Pt. 58 68°C). The product is finally vacuum dried during which the residual solvent is also removed. The concentrate prepared by this method contains 85% protein of which 96% is pepsin digestible. The product is practically odourless and almost white in colour.

INTRODUCTION

Protein malnutrition as is being experienced all over the world has been attracting the attention of nutritionists and others since quite sometime and much effort has already been expended in this field. Production of inexpensive and at the same time nutritious food can do much to tackle this problem and free the millions out of this grave situation. In the opinion of experts fish is considered to be the ultimate answer to this burning problem mainly because of its abundance in nature, cheapness and high nutritive value. The main consideration is how to make fish which is highly perishable useful for the purpose. The importance this problem has attained is evident from the numerous publications on the subject and the interest shown by world organisations.

Of the annual fish landings in India, considerable amount is constituted by cheap variety of fishes of miscellaneous types, which find no ready response from consumers. In a country like ours, where the problem of protein malnutrition is really serious, conversion of the above fish to edible flour will be of much use. Work done in India on utilization of surplus fish has mainly been confined to production of fish meal. The main attempts made to prepare edible flour from fish consisted in the works of Pillai (1956 and 1957), Bhatia, Moorjani, Iyengar and Visweswaraiah (Bhatia et. al, 1955), Moorjani and Lahiri (Moorjani et. al, 1961), Moorjani, Bala-

krishnan Nair, Krishnaswamy and Lahiry (Moorjani et. al, 1962), Kadkol and Lahiry (Kadkol et. al, 1962) and Revankar, Khabade and Suryanarayana Rao (Revankar et. al, 1965).

The methods practised elsewhere based on solvent extration e. g. French Process, Viobin Process, Canadian Process etc. are not free from draw backs. In extraction by acetone, difficulty is experienced to remove traces of acetone even under reduced pressure. Moreover acetone is less efficient in lipid liberation and will extract substances other than lipids (Moorjani and Lahiry 1962). In Viobin process, which employs the principle of azeotropic extraction with ethylene dichloride, Morrison and Munro (Morrison et.al, 1965) showed that ethylene dichloride destroys cystein, histidine and interfere with the release of cystein, histidine and methionine by pancreatic digestion. Another important process employed for the preparation of edible fish flour is the Canadian process which employs isopropyl alcohol for the extraction; but the flour retains a solvent taint even after prolonged steam stripping under vacuum (Moorjani etal 1962). The process was later modified and extended to the preparation of edible fish protein concentrate from whole fish by the Bureau of Commercial Fisheries U. S. A. (1966). MIT-UNICEF process described by Allen (1963) employs hexane and alcohol for the extraction of fat.

The present paper describes a modification of the MIT-UNICEF process and establishes the advantages of using an azeotrope of hexane and alcohol for the extraction of fat.

Materials and methods

Fish for the experimental work were collected from the catches of trawlers operated off Cochin by the Institute and kept iced till used for preparation of Fish Protein Concentrate. The fish after washing in potable water were processed in four different ways.

(1) Flesh alone taken after removal of head, viscera, scales bones etc. from individual species.

(2) Discarding only head, viscera, and scales and retaining the bones from individual species.

(3) Employing a mixture of different trash fishes comprising mainly of jew fish (*Pseudosciaena Spp*) arana (*Saurida tumbil*), silver belly (*Leiognathus Spp.*) and malabar sole (*Cyanoglossus Spp.*) after dressing as in (2).

(4) Employing a mixture of different trash fishes comprising the same species as in (3), without dressing.

Analytical methods

The composition of fish flour as regards the moisture, fat, nitrogen, ash and pepsin digestibility were determined by the methods of AOAC (1960) and the available lysine was estimated by Carpenter's method (1960).

Preparation of the flour

The fish after mincing is suspended in an equal amount of water to which has been added 0.5% acetic acid and kept at 70—80°C. for 30 minutes under constant stirring. The lurry is filtered and pressed in a screw press so that a good amount of liquid is removed. The press cake will contain nearly 60—62% moisture It is then dried in a tunnel dryer at a temperature range of 75—80°C. to various levels of moisture. The dry matter (moisture content below 10%) is coarsely ground in an ordinary milling machine and used for extraction of fat.

Extraction

The material is charged in to the extra-

ction drum having arrangements for stirring and refluxing. The solvent, azeotropic mixture of hexane and ethyl alcohol (33.2 mole per cent alcohol b. p. 58.68°C) is added in two parts by volume to one part by weight of material and kept at the boiling temperature of the solvent for 30 minutes, under constant stirring. The solvent is drained off and fresh solvent added in 1.5 parts by volume to one part by weight of original material and refluxed under constant stirring for 30 minutes. After draining off the solvent the process is repeated once again. After final draining the adhering solvent is distilled off and the last traces removed by steam stripping under reduced pressure. The dried matter is pulverized to fine mesh size using a milling machine.

In the course of the experiments data were also gathered on the effect of various concentrations of acid added to the minced fish during the pre-cook stage on the final product, on the relative rates of extraction of fat by different solvent systems and on the effect of temperature and mode of extraction on rate of defatting and deodourization by the azeotropic mixture of hexane and alcohol.

Results and discussion

In all the trials on acidification, acetic acid was used although polyphosphoric acid (Power 1962; and Guttmann et. al, 1957) and hydrochloric acid (Pariser et. al, 1959) have been mentioned by other workers. The effect of different concentrations upto 1.5% on the minced fish is given in Table I. It is seen that acidification beyond a concentration of 0.5% (W/V) produces an adverse effect on the digestibility of the protein. Although the odour of the product is considerably reduced in the acid treated press cake, which is in agreement with the observations of Guttmann and Vandenheuvel (1957) the product acquires a deeper colour with increasing concentration of acid beyond 0.5%.

In their efficiency for removal of fat, all the solvents except ethyl alcohol are more or less equally effective (Table II) and all of them bring down the fat level below 0.75%, which is in conformity with the standards prescribed by FAO (1962). Acetone and isopropyl alcohol extraction is found to result in a slightly higher fat content compared to other solvents used in the experiments. Hexane alone is not effective in deodourizing the flour as it is a poor extractant for bound lipid fraction which comprises largely of phospholipids (Moorjani et. al. 1962), although it is found to be the most efficient single solvent for fat extraction and alcohol alone is insufficient for the removal of fat but is the most efficient solvent for liberating and extracting bound lipids (Moorjani et. al. 1962). The azeotropic mixture however, is found to satisfy both needs, almost complete removal of odour and bringing down the fat content to around 0.3%.

The ratio of the solvent to the dry material, extent of extraction of fat at 29°C., 45°C. and at the boiling point at intervals of 30 minutes during continuous and batch extraction are given in Table III. The extraction rate increases with increase in temperature and period, the extraction being more in batch process by changing the solvent after every 30 minutes, the residual fat content after 1½ hours extraction being only around 0.25% whereas 3.68% fat is retained in continuous extraction for the same period.

Azeotropic mixture of the two solvents – hexane and alcohol – has been observed to have many advantages. It boils at a temperature lower than the boiling point of either hexane or alcohol.

It is found to be miscible only at the boiling point and the mixture after extraction and draining can be distilled off completely free from fat, which on cooling separates into two layers, making the further purification of alcohol easy. Alcohol which will contain the odouriferous matter can be purified either by refluxing with acid and alkali respectively followed by distillation or by treatment with activated carbon.

In order to find out the possibility of subjecting the press cake after acid treatment for direct extraction with azeotropic mixture at different moisture levels of press cake was studied and the data are presented in Table IV. It is evident from the table that there is no difference in the extent of extractability at 67% and 20% moisture, difference becoming significant only at 7% moisture level. This indicates that partial drying has no special advantage. This led to a possible prediction that pess cake can be extracted even before drying, which was confirmed in actual trials carried out (Table IV). By avoiding drying of the press cake the colour of the finished product also was found to be improved, which is considered to be a definite advantage apart from the savings in labour and time. This procedure might also probably remove the possibility of secondary oxidation products of fat and formation of polymerisation products which are known to be toxic. (Privett 1959, Venolia et. al, 1958, Kaunitz et. al, 1956) the former of which can destroy vitamins and in the case of the latter it has been reported that formation of co-polymers with protein may effect reduction in the content of available lysine (Lea et. al, 1960). The data presented in the table also show that alcohol alone does not efficiently remove fat in 30 minutes extraction. However, when this was followed by an extraction with zeotropic mixture for 1½ hours the effect is very significant, the fat content being brought down to 0.53% as against a fat content of 1.01% in a 2 hour extraction with azeotropic mixture alone.

From the data presented in Table VI it is evident that apart from the difference in the protein and ash contents which could be accounted for by the presence of bones in the sample, there is no significant difference between samples prepared from individual type of fish used in the form of flesh alone or from dressed fish or from whole fish regarding digestibility and available lysine content. All the samples were free from any objectionable odour and taste but the colour of the latter was found to be deeper yellow.

Summary

Possibility of preparation of edible fish flour from the commercially available and cheap varieties of fish is dicussed. Effect of different solvents on the extraction of fat from press cake after drying and also modification of MIT-UNICEF method using azeotropic extraction of fat has been worked out. Possibility of using wet press cake directly for extraction by modification of this using extraction by alcohol prior to extraction by azeotropic mixture has been discussed. A comparison has been made of the fish flour prepared from fish muscle alone, dressed fish (of the same species), mixed dressed fish and from whole fish.

Acknowledgement

The authors are grateful to Dr. A. N. Bose, Director of the Institute, for his keen interest and valuable suggestions during this study.

References

Allen L. E. 1963. *Fising News International*, **2**, 1, 106.

Bhatia D. S., Moorjani M. N., Iyengar J.R.

TABLE I EFFECT OF ACID ON THE QUALITY OF FLOUR.

Analytical Characteristics %	Percentage of acid used			
	0.00%	0.5%	1%	1.5%
Moisture	7.81	7.75	6.74	6.56
Ash	21 87	21.13	20.25	17.57
Fat	9.14	9.52	10.77	11.63
Protein	64.44	62.94	61.37	59.87
Pepsin Digestibility	96.08	96 12	93.80	91.20

TABLE II EFFECT OF DIFFERENT SOLVENTS ON EXTRACTABILITY OF FAT.*

Time in min.	Wet press cake		Dried powder		
	Isoproponal	Acetone	Alcohol	Hexane	Hex--Alcohol
30	7.44	8 53	8.63	7.51	5.75
60	4.24	4.14	6.65	4.12	1.49
90	2.89	2.84	5.02	0.81	0.43
120	0.44	0.71	4.82	0.12	0.30

* The figures represent the residual fat (%) in the finished flour.

TABLE III EFFECT OF TEMPERATURE AND MODE OF EXTRACTION OF FAT CONTENT

Solvent Ratio	Mode of Extraction	Time in min	Residual fat content at intervals with temperature (%)			Recovery of solvent with temperature (%)		
			29°C	45°C	58.68°C (Boiling point)	29°C	45°C	58.7°C
1:2	Continuous	30	7.31	6.37	5.06			
		60	4.62	5.77	3.95			
		90	4.41	4.75	3.68	92.8	91.1	93
		120	4.16	4.20	3.22			
1:2 in the first extraction and 1:1.5 in subsequent extraction	Batch-wise	30	5.75	3.96	2.89			
		60	1.49	0.67	0.40			
		90	0.43	0.28	0.26	93.7	92	91.8
		120	0.30	0.16	0.08			

TABLE IV EFFECT OF MOISTURE CONTENT OF PRESS CAKE ON FAT EXTRACTION

(Temp. 58.68°C. Batchwise, (1.2 in the first extraction, 1:1.5 in subsequent)

Moisture %	Time in min.	Residual fat content (%)	Solvent recovery %
67	30	4.74	86.4
	60	4.54	
	90	1.09	
	120	1.01	
47	30	4.63	90
	60	4.17	
	90	1.56	
	120	0.83	
20	30	4.59	90
	60	4.09	
	90	1.22	
	120	0.41	
7	30	2.89	91
	60	0.67	
	90	0.26	
	120	0.08	

TABLE V COMPARISON OF EXTRACTION BY VARIOUS SOLVENTS ON WET PRESS CAKE

Time in min.	Percentage of residual fat (dry basis)		
	Alcohol Hex.	Alcohol Azeotrop.	Alcohol followed by Azeotrop
30	8.63	4.74	2.08
60	6.65	3.54	1.00
90	5.02	1.09	0.54

TABLE VI ANALYTICAL CHARACTERISTICS OF THE DIFEERENT SAMPLES

Description of the material	Moisture %	Ash %	Fat residual %	Protein %	Pepsin digestibility %	Available lysine gm/16gm of nitrogen
Flour from the muscle alone	7.16	6.48	0.26	84.87	96.6	8.73
Flour from dressed fish	7.15	21.03	0.38	72.64	94.1	7.81
Flour from dressed miscellaneous fish	7.50	18.11	0.24	73.17	94.5	7.84
Flour from mix. fish muscle alone wet extraction	6.94	4.78	0.54	87.50	95.9	8.12
Wet press cake misc. dressed fish	6.40	14.36	0.59	79.69	93.8	7.71
Flour from whole fish wet extraction	7.22	23.11	0.51	68.24	93.4	7.51

and Visweswaraiah K. 1959. *Food Sci.* *8*, 1.

Bureau of Commercial Fisheries 1966. Fishery Leaflet 584.

Carpenter K. J. 1960. *Biochem. J.*, **77**, 604.

FAO Fisheries Report 1962, No. 21962. p. 17.

Guttmann A. and Vanderheuvel F. A. 1957. *Prog. Repts. Atl. Coast. Sta.*, F. R. B. C; No.67. pp. 21-31.

Kadkol S. B. and Lahiry N. L. 1962. *J. Sci. Ind. Res., 21 D,* 386.

Kaunitz H , Slantz C. A., Johnson R. E., Knight H. B., Sanders D. H., and Swern D. 1956. *J. Am. Oil. Chem. Soc.*, **33**, 630.

Lahiry N. L., Moorjani M. N., Visweswaraiah K., Shurpalekar S. R., Swaminathan M., Srinivasan A. and Subrahmanyan V. 1962 *Food Sci.*, **11**, 2, 37.

Lea C. H., Parr L. J. and Carpenter K. J 1960. *Brit. J. Nutr.* **14**, 91.

Moorjani M. N., Balakrishnan Nair R., Krishnaswamy M. A. and Lahiry N. L. 1962. *Res. and Ind.*, **7**, 307.

Moorjani M. N. and Lahiry N. L. 1961. "Symposium on Proteins". CFTRI. Mysore.

Moorjani M. N. and Lahiry N. L. 1962. *Reviews in food Sci. and Tech.*, **4**, pp. 113-136.

Moorison A. B. and Munro I. C. 1965. *Canadian J. Biochem.* **43**, 33.

Pariser E. R. *et. al.* 1959. Mimeographed Report for UNICEF Dept. of. Food. Technonology MIT.

Pillai V. K. 1956. *Curr. Sci.*, **25**, 293.

Pillai V. K. 1957. *Res. and Ind.*, **2**, 265.

Power H. E. 1962. *J. Fish. Res. Bd. Canada.* **19**, 1039,

Privett O. S. 1959. *J. Am. Oil. Chem. Soc.*, **36**, 507.

Revankar G. D. Khabade V. S. and Suryanarayana Rao S. V. 1965. *Res. and Ind.*, **10**, 12, 364.

Venolia A. W. and Tappel A. L. 1958. *J. Am. Oil. Chem. Soc.*, **35**, 135.

Solvents for the Flavor Industry

No single solvent can be utilized for all purposes by the flavor industry for certain solvents are better for some purposes than others

Am. Perfumer Essent. Oil Rev. 47 (10): 53-4, (1945)

by MORRIS B. JACOBS, PH.D.

THE search for the ideal solvent for flavors still goes on. It is questionable whether the search for a single solvent for all purposes is worthwhile. Almost every solvent employed by the flavor industry has certain advantages and certain disadvantages. Thus even ethyl alcohol has the disadvantage of high cost and high volatility although the latter factor is at times an advantage. Thus in the case of flavoring of powders the volatility of ethyl alcohol assists in its rapid evaporation leaving the powder dry.

GLYCEROL

Glycerol, $HOCH_2CH(OH)CH_2OH$, glycerin, trihydroxypropane, is a clear, colorless, odorless, hygroscopic sirupy liquid, with a sweet, warm taste. It has a specific gravity of 1.262-1.265, boils at 290 deg. C. with some decomposition and has a refractive index of 1.1473. It is miscible with water, alcohol, and propylene glycol. One volume of glycerol is soluble in 11 of ethyl acetate. U.S.P. glycerol must contain not less than 95 per cent of this substance. It is not very soluble in many common organic solvents nor are many organic substances readily soluble in it.

Glycerol has been obtained by purely synthetic means, but its principal commercial source is as a by-product in the manufacture of soaps and fatty acids from oils and fats. It is purified by distillation.

Because of its sweet flavor and solvent properties glycerol was, and is used widely as a solvent for flavoring materials. It has been employed extensively in perparation of genuine and imitation vanilla extracts. Since it is hygroscopic, it is employed as a humectant or moistening agent in bakery products and in confectionery, principally to prevent the drying out of such products as icings and cakes. Glycerol has some preservative action against mold and hence is used in candy both as a preservative and as an additional sweetening agent. The high viscosity of glycerol is at times a disadvantage, particularly in its addition to a mixture, and at other times an advantage as in lending smoothness to a mixture.

PROPYLENE GLYCOL

Propylene glycol, CH_3CHOH-CH_2OH, 1,2-propanediol, is a colorless, practically odorless, hygroscopic, somewhat viscous liquid. It has a specific gravity of 1.035-1.040, boils at 189 deg. C. and has a refractive index of 1.4300-1.4310 at 25 deg. C. The commercial product boils in the range 185-190 deg. It is miscible with water, alcohol, glycerol and many of the common organic solvents.

The Division of Pharmacology of the Food and Drug Administration conducted extensive investigations on both the acute and chronic toxicity of propylene glycol. Their review of available results shows that, in the amounts normally used in food products, propylene glycol is to be regarded as a harmless ingredient.[1] Consequently, this Federal Agency has decided not to interpose any objection to the replacement of glycerol or other solvent by propylene glycol in food products from the standpoint of the requirements of the Federal Food, Drug and Cosmetic Act. Because of this permission, the use of propylene glycol in flavor products has increased in recent years.

The neutral flavor properties and the solvent powers of propylene glycol permit it to be used in a variety

of different ways. Thus, if it is used to dissolve synthetic flavoring materials the addition of some water may still be possible. Because of its ability to lower surface tension propylene glycol assists in distributing evenly the flavoring materials used. Because it is hygroscopic, it can be employed as a humectant in confectionery and bakery products. Propylene glycol has some preservative action. In flavoring materials it serves to prevent mold growth and is better for this purpose than glycerol.

TRIACETIN

Triacetin $(CH_3COO)_3C_3H_5$, glyceryl triacetate, is an odorless, colorless, oily liquid which boils at 258-259 deg. C., has a specific gravity of 1.156-1.161 and a refractive index of 1.4306. One volume is soluble in 14 of water and it is miscible with alcohol. Because of its neutral flavor qualities and its solubility properties it can be used as a solvent and also as a fixative. Gazan[2] details many flavor compositions in which triacetin acts as the solvent.

Glyceryl monoacetin resembles glycerol in its solubility properties and *diacetin* resembles triacetin. There are two principal objections to the use of the acetins as flavor solvents. In the first place they tend to hydrolyze to yield glycerol and acetic acid giving the flavor mixture an acetic acid odor and secondly they impart an off-taste to the flavor.

ISOPROPYL ALCOHOL

Isopropyl alcohol $(CH_3)_2CHOH$, 2-propanol, is a colorless liquid with an odor which is somewhat harsh compared to ethyl alcohol and which has some resemblance to acetone but with a fruity note. It has a specific gravity of 0.783-0.789, a boiling point of 82.5 deg. C. and a refractive index of 1.3780. It is completely miscible with both water and alcohol. Isopropyl alcohol can be synthesized by the reduction of acetone or hydration from propylene which is a product of the cracking of petroleum. While the principal use of isopropyl alcohol is that of a solvent, it has been suggested for use in small amounts as a flavor component in apple, apricot, banana, currant, gooseberry and raspberry flavors.

In the ratio of 1 ounce per gallon of carbonated water, isopropyl alcohol has only a slight taste. Its odor, however, tends to mask the odor of other components of an essence hence it does not have the blending ability of ethyl alcohol. Isopropyl alcohol has had some advocates as a flavor solvent. It has, in common with aliphatic alcohols, a narcotic action. Isopropyl alcohol is not considered extremely toxic and it resembles in some measure ethyl alcohol in its physiological effect but is more harmful. The toxicity of the aliphatic alcohols has been reviewed by Oettingen.[3]

However, the U. S. Food and Drug Administration has stated that while pharmacological investigations have not established the relative toxicity of isopropyl alcohol, they feel that its properties are not sufficiently well-known to permit regarding it as suitable for use in colors which are to be certified.[4] This would imply that its use as a flavor solvent is also regarded with disfavor.

OTHER SOLVENTS

While propylene glycol and glycerol are considered safe components for preparation of flavoring essences, the Food and Drug Administration considers ethylene glycol, carbitol and diethylene glycol harmful.[5] This Agency states that these compounds are so harmful that their presence in excess of 5 per cent for topical application to small areas of the body would constitute a hazard.

MONOETHYL ETHER

The ethyl ethers of glycerol,[6] particularly monoethyl ether have been proposed as solvents for the preparation of flavoring extracts. They are stable, miscible with water, do not hydrolyze, have relatively high boiling points and therefore can serve as fixatives, and are said to show exceptionally good solvent power for the various essential oils.

EDIBLE OILS

Odorless, bland and tasteless edible oils have been used as solvents for flavoring materials particularly for the preparation of products for the bakery trade. Such products have a relatively well established position. They tend, however, to become rancid with time. This is a serious disadvantage. Furthermore, only "winterized" oils should be used to avoid precipitation of fats at low temperatures. Mineral oil has been used, at times, but is not regarded favorably.

While *ethyl acetate* (acetic ether) is considered a principal flavor component it may also be considered a flavor solvent for its solvent function as that of a blending component.

Acetic acid is used as a solvent for essences prepared for meat products. *Benzyl benzoate* is a solvent used for the artificial musks used principally for berry flavors.

REFERENCES

[1] U. S. Food, Drug Admin. FD&C Act Trade Correspondence TC-374, December 10, 1941.

[2] M. H. Gazan, "Flavours and Essences," Van Nostrand, New York, 1936.

[3] W. F. von Oettingen, U. S. Pub. Health Service, Pub. Health Bull. No. 218, 1943.

[4] U. S. Food, Drug Admin. FD&C Act Trade Correspondence TC-162, March 14, 1940.

[5] U. S. Food, Drug Admin. FD&C Act Trade Correspondence TC-402, May 14, 1943.

[6] U. S. Patent 2,180,932, Nov. 21, 1939.

Flavored Notes

Essential oils, flavoring extracts and essences should be protected from light by storing in colored bottles, particularly chlorophyll green bottles, although brown bottles also prove to be suitable. Light accelerates the decomposition of these materials.

* * *

Flavoring materials and essential oils are readily affected by oxidation. Consequently, it is good practice to keep the amount of air above these substances in their containers at a minimum. If a bottle of material has been partially used it is best to transfer the contents to a smaller bottle.

* * *

Pastes can be prepared in such viscous form that they can be pressed into segmented plastic blocks, each segment having sufficient flavoring material to flavor a given weight of food or candy.

* * *

Soft cakes containing chocolate or cocoa have their best flavor when the pH of the finished product is 7.5-7.8

M. B. J.

Appetite Regulation in the Ruminant

Don R. Jacobson, Scott B. Carr, and *Toha Sutardi,* Department of Animal Sciences, University of Kentucky, Lexington

Introduction

A knowledge of appetite or more specifically voluntary food intake regulation has recently come to be rather widely recognized as being of fundamental importance to the improvement of health and longevity in many animal species as well as to the improvement of animal performance in the production of food for the human being. A further consideration of great economic importance in ruminant animals is the need for an understanding of the factors that regulate voluntary feed intake so that increased feed intake over a wider range of ingredients may be obtained which would allow for more efficient production of milk and meat. Some investigators have considered voluntary feed intake to be a function of certain "physical" parameters such as "rumen fill" or "rate of passage" of digesta through the gastro-intestinal tract. Others have considered voluntary feed intake in the ruminant to be largely influenced by intermediary metabolites which has been referred to as "chemical". Dowden and Jacobson [1] first presented data showing that chemicals may be involved.

A. Voluntary Feed Intake and Body Weight or Milk Production

Nineteen lactating Holstein cows were fed only good quality alfalfa hay *ad libitum* for five weeks in order to obtain data for the purpose of measuring the degree to which milk production, body weight and barrel circumference were correlated with voluntary dry matter intake. The single gross correlations of each of these measurements with dry matter intake were 0.11, 0.17, and 0.16, respectively. Multiple correlation of milk production and body weight with dry matter intake was 0.56. This value was highly significant ($P < 0.01$). However, 69 % of the variation in intake was not accounted for. These results are in general agreement with those of Legates et. al. [2].

B. Mass Added to the Rumen or Removal of Rumen Contents on Voluntary Feed Intake

In the first trital, inert polyethylene cubes comprised 4.0, 7.5, 13.5, and 22.6 % of the total ration consumed including long hay. Four sets of identical twins of dairy breeding were employed in a single reversal design with two pairs of twins receiving each of the treatment levels. Three of the four animals did not readily accept the 22.6 % cube ration. The voluntary feed intakes for the different levels of polyethylene as given above respectively were −0.4, −0.5, −0.6, and −3.3 kg. The two higher levels caused a significant reduction in nutrient intake. Total intake, however, was increased in all groups except the last one (Table I). The data are interpreted to mean that mass or bulk added to the diet is of minor importance in determining the voluntary feed intake. At the very high levels (13.5 and 22.6 %) the animals discriminated against the cubes so as to cause a reduction in nutrient intake.

In the next three trials the effect of additional inert mass placed in the rumen on voluntary feed intake was studied. Mass (containerized polyethylene cubes or water) was added to the rumen at levels of 1.6, 4.9, and 9.2 % of metabolic size (kg body wt.$^{0.75}$). Chopped alfalfa hay was offered *ad libitum* to 6 or 8 fistulated animals for 3 hours each afternoon.

Five-day experimental periods were employed with a cahnge-over period prior to the reversal of the experimental groups.

Table I. Voluntary intake when polyethylene comprised from 4.0 to 22.6 % of the consumed ration

	Per cent polyethylene in consumed ration			
	4.0	7.5	13.5	22.6
	(kg/animal/day)			
Feed intake of control group[1])	12.7	11.3	10.0	12.9
Feed intake of treatment group	12.3	10.8	9.4	9.6
Total intake of treatment group	12.8	11.7	10.9	12.4
Difference in feed intake	—.4	—.5	—.6[2])	—3.3[2])
Difference in total intake	0.1	0.4	0.9[2])	—.5
Polyethylene consumed	0.5	0.9	1.5	2.8
	%			
Change in feed intake	3.1	—4.4	—6.0	—25.6
Change in total intake	0.8	3.5	9.0	—3.9

[1]) Each group comprised of four animals.
[2]) Differences between treated and control groups were significant at $P < 0.05$.

Mass added to the rumen at any of the three levels caused no significant effect on voluntary feed intake (Table II).

Table II. Mean and adjusted mean dry matter intake of chopped hay when containerized additional mass was placed in the rumen

Additional mass added	Number animals per group	Group[1])	Dry matter intake kg	Adjusted intake[2]) kg
Polyethylene at 1.6 % kg body wt $^{0.75}$	8	Control	8.5	8.1
		Treated	8.2	7.6
		Difference	—.3	—.5
Water at 4.9 % kg body wt $^{0.75}$	8	Control	8.4	7.9
		Treated	7.9	7.5
		Difference	—.5	—.4
Water at 8.2 % kg body wt $^{0.75}$	6	Control	9.4	9.2
		Treated	9.3	9.1
		Difference	—.1	—.1

[1]) Differences in intake between treatment and control groups were not significant.
[2]) Individual dry matter intakes were adjusted to that of a 500 kg animal proportional to kg body wt $^{0.75}$.

In the last three trials, ingesta was removed at 1.6, 4.9 % levels via the fistula prior to the feeding period and at 8.2 % three hours after feeding began. The two lower levels had no effect on voluntary feed intake, but the higher level did (Table III). These data suggested

that "physical" fill is not a limiting factor in voluntary feed intake, whereas the removal of rumen contents after feeding which caused the animals to increase their feed intake is taken to mean that there is "chemical" influence.

Table III. Mean and adjusted mean dry matter intake of chopped hay when rumen contents were removed from the rumen

Amounts removed	Number animals per group	Group	Dry matter intake	Adjusted intake[1])
1.6 % kg body wt $^{0.75}$ removed before feeding	6	Control	9.0	8.3
		Treatment	8.7	8.0
		Difference	0.3	0.3
4.9 % kg body wt $^{0.75}$ removed before feeding	8	Control	9.4	8.4
		Treatment	9.5	8.6
		Difference	0.1	0.2
8.2 % kg body wt $^{0.75}$ removed after feeding	6	Control	9.4	9.2
		Treatment	9.9	9.6
		Difference	0.5[2])	0.4[2])

[1]) Individual dry matter intakes were adjusted to that of a 500 kg animal.
[2]) Differences between control and treatment groups were significant at $P < 0.05$.

C. Voluntary Feed Intake and Blood Levels of Certain Metabolites Following Intravenous Infusion of Acetate and Propionate

Sixteen dairy steers were used in one preliminary and three trials to study the effect of continuous infusion of either acetic or propionic acid upon voluntary feed intake and blood and rumen levels of volatile fatty acids. Infusions were in 3 liter volume for 8 hours duration for 3 consecutive days to supply up to 10 % of the digestible energy requirement. Control animals received the same volume of saline solution under the same conditions.

Table IV. Voluntary feed intake per 453.6 kg$^{0.75}$ animal during and after intravenous infusion of treatment animal of each pair with acetic acid

Animal pair no.	Level of infusion[1])	Voluntary feed intake 8 hr[2])			16 hr[2])			24 hr[2])		
		T	C	D	T	C	D	T	C	D
1	10	2.45	3.86	—1.41	3.86	4.72	—0.86	6.31	8.58	—2.26
2	10	0.54	3.86	—3.32	4.94	4.72	0.22	5.49	8.57	—3.08
3	10	1.00	4.40	—3.40	4.58	4.45	0.13	5.58	8.85	—3.27
4	10	0.45	1.91	—1.46	1.18	3.54	—2.36	1.63	5.44	—3.81
5	6.25	0.18	2.72	—2.54	3.86	2.86	1.00	4.04	5.58	—1.54
6	2.7	2.36	2.58	—0.22	6.17	5.58	0.59	8.53	8.16	0.37
7	2.5	3.63	2.90	0.73	6.26	5.03	1.23	9.89	7.94	1.95
8	0	1.77	2.36	—0.59	2.72	3.81	—1.09	4.49	6.17	—1.68
0	0	2.59	3.40	—0.81	6.17	6.08	0.09	8.75	9.48	—0.73

[1]) Expressed as percentage of estimated digestible energy requirement.
[2]) Difference due to infusion at 6.25 to 10 % level statistically significant ($P < 0.01$).
[3]) T, C, and D refer to treatment, control, and difference, respectively.

The infusion of acetic acid (not adjusted to pH 7.0) reduced significantly ($P<0.01$) the voluntary intake of chopped alfalfa hay of the treatment animals during the days of the infusion and during the 4-day recovery period (Table IV). Acetic acid (adjusted to pH 7.0) infusion at comparable levels appeared to have less effect on voluntary feed intake.

Infusion of acetate or propionate caused an increase in the blood level of the respective metabolites. In addition, acetate infusion led to an increase in blood butyrate and sometimes an increase in blood propionate. Infusion of propionate caused a reduction of blood acetate, but had no effect on the blood butyrate level. There was a significant multiple correlation between blood levels of acetic, propionic, and butyric acids and level of infusion and voluntary feed intake. These observations suggest that the short term mechanism of feed intake regulation is not dependent upon one metabolite alone, but rather upon an interrelationship of at least the three most important volatile fatty acids. The zero hour levels of metabolites which were considered to be normal were significantly correlated with voluntary feed intake. Much of the variation of the voluntary feed intake in the entire study could be accounted for by blood levels of metabolites and by levels of infusion.

9. Blood and Rumen Volatile Fatty Acid Levels of Cows on a Special Diet Where Extremely High Voluntary Intakes Occurred

Six group comparison lactation trials of 6 to 12 weeks each employing 18 to 24 Holstein and Jersey cows on equal and constant grain levels were conducted over a 5 year period by comparing variations of high population (HP) corn silage with standard plant (SP) corn silage. The high population corn silage was harvested by July 1 from fields that

Table V. Rumen and jugular blood VFA levels at three to four hours after feeding for two different corn silages

Week of experiment / Silage	5 SP [1])	5 HP - 1 H [2])	8 SP	8 HP - 1 H
Rumen contents		M Moles / L		
Acetate	54.9	63.7 [3])	56.2	61.2 [3])
Propionate	16.1	16.5	17.0	15.4 [3])
Butyrate	11.0	11.6	10.9	11.1
		Molar %		
Acetate	62.9	65.5	63.2	66.0
Propionate	18.6	17.1 [3])	19.2	16.5 [4])
Butyrate	12.6	11.9 [3])	12.3	11.9
C_2 / C_3	3.43	3.84 [3])	3.33	4.02 [4])
C_2 / C_4	5.02	5.50 [3])	5.20	5.58
Blood		M Moles / L		
Acetate	2.66	3.01 [3])	2.21	2.70 [3])
Propionate	0.14	0.14	0.10	0.09
Butyrate	0.04	0.03	0.03	0.04

[1]) Standard plant corn silage.
[2]) High population corn plus 200 kg soybean hulls per metric ton corn as ensiled.
[3]) Statistically significant ($P < 0.05$).
[4]) Statistically significant ($P < 0.01$).

contained from 74–740 × 10^3 plants per hectare. In trial five, voluntary silage dry matter intake was increased by 4.4 kg per cow per day and milk production was increased 3.1 kg per cow per day over the control group by the eighth week of the trial. Associated with this remarkable and previously unattained increase in voluntary dry matter intake and milk production were significant changes in the volume and blood volatile fatty acid picture (Table V). Rumen acetate and propionate, as well as the C_2/C_3 molar percent ratio, increased.

These data are not easily reconciled with the view that increased acetate in the blood stream at threshold levels inhibit voluntary feed intake. On the other hand, the only blood samples analyzed were from the jugular vein taken at three to four hours after feeding. It is possible that acetate or its degraded or converted product in the arterial or portal supply at time other than three to four hours after feeding is critical.

References

[1] Dowden, D. R., and Jacobson, D. R.: Inhibition of Appetite in Dairy Cattle by Certain Intermediate Metabolites. Nature, Lond., 188, 148 (1960).

[2] Legates, J. E., Murley, W. R., and Waugh, R. K.: Hay Consumption of Individual Cows on Limited Grain Feeding. J. Dairy Sci., 39, 937 (1956).

Gas-liquid Partition Chromatography: the Separation and Micro-estimation of Volatile Fatty Acids from Formic Acid to Dodecanoic Acid

By A. T. JAMES and A. J. P. MARTIN
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 5 *June* 1951)

Industry has long used charcoal or other solid adsorbents in columns for chromatographic for solvent recovery, and more recently Turner (1949), Cherry (1949), Glueckauf, Barker & Kitt (1949), Phillips (1949) and Tiselius (1950) have used charcoal in gas chromatograms for the analysis of hydrocarbons and esters. Gas-liquid scrubbing columns have been used in industry for many years but, though Martin & Synge (1941) suggested the use of gas-liquid partition chromatograms for analytical purposes, no work has been reported above these lines.

This paper presents modifications necessary to the theory of Martin & Synge (1941) to allow for the compressibility of the mobile phase and describes the application of the gas-liquid partition chromatogram to the separation of volatile fatty acids. The separations obtainable by this method are essentially parallel to the so obtainable by distillation, but good separations can be achieved much more easily and it is possible to work with very much smaller quantities. In fact, the lower limit of quantity of material used is determined only by the efficiency of detection. In general, for less trouble from azeotropic fractionation is to be expected, since the concentrations of the substances to be separated in the liquid phase are always low, and it may be possible to choose a liquid phase which associates only with one component of the azeotrope. Work on a preparative scale, though theoretically possible, is likely to be inconvenient because of the bulk of the apparatus. The method of detection described here is acid-base titration, but many other methods of detecting changes in the composition of a gas stream could be used and it is intended in future publications to describe some of these, which should extend the range of application to all substances capable of being distilled at the pressure of a few mm. of mercury.

In suitable cases the gas-liquid partition column has several advantages over the so-called liquid-liquid partition column: (*a*) the low viscosity of the mobile phase allows much longer columns to be used with a corresponding increase in efficiency and (*b*) it is easier to obtain a constant phase in composition of a gas than of a liquid stream.

A THEORY OF GAS-LIQUID PARTITION CHROMATOGRAPHY

The theory of gas-liquid chromatography differs from that of liquid-liquid chromatography, where a constant partition coefficient is assumed (Martin & Synge, 1941), only by virtue of the fact that the mobile phase is compressible and that it has a gradient of pressure along the column. It is assumed that the partial pressures of the substances to be separated are negligible in relation to that of the carrying stream of gas. Let

P_1 = pressure of gas applied to chromatogram.
P_0 = pressure of gas at outlet.
P = pressure of gas at point distant x from the outlet.
v = linear velocity of gas at point x.
l = length of column.
t = time which elapses before the centre of the zone emerges from the column.
F = volume of gas emerging from outlet in unit time.
V_P = tF = the retention volume of the centre of the zone.
V_P^0 = limiting value of V_P as P_1/P_0 tends to unity.
a = the area occupied by the gas phase in any cross-section of the column.
K = column constant, a function of the viscosity of the gas phase and the tightness of the column packing.

Then
$$K\frac{dP}{dx} = \frac{FP_0}{P} = av.$$

Integrating to give the pressure along the column:

$$K\frac{P^2}{P_0} = 2Fx + KP_0.$$

or
$$K = \frac{2FxP_0}{P^2 - P_0^2}.$$

or
$$F = \frac{P_0K}{2x}\left[\left(\frac{P}{P_0}\right)^2 - 1\right]. \qquad (1)$$

Now
$$t = \int_0^l \frac{dx}{v} = \int_0^l \frac{a\,dx}{F_P P_0 P^{-1}} \qquad (2)$$

Here R_F has the usual chromatographic meaning (Consden, Gordon & Martin, 1944), that is, it represents the rate of movement of the zone of the substance under consideration relative to the rate of flow of the moving phase. In the present case the rate of flow of the moving phase increases continuously as it advances down the column, but the R_F remains constant and the rate of movement of the zone increases correspondingly. The R_F is a constant depending only on the substance to which it refers, the temperature, the nature and amount of the stationary phase and, in so far as the support is not inert, on its amount.

Rewriting Eqn. 2 in terms of pressures,

$$t = \int_{p_0}^{p_1} \frac{Ka\,p^2\,dp}{R_F\,P^2\,p_0^2} = \frac{Ka\,(p_1^3 - p_0^3)}{3R_F\,p_0^2\,P},$$

$$V_R = Ka\,p_0\,\frac{[(p_1/p_0)^3 - 1]}{3R_F\,P}.$$

Hence, by (1),

$$V_R = \frac{2\,al}{3\,R_F}\,\frac{(p_1/p_0)^3 - 1}{(p_1/p_0)^2 - 1}. \qquad (3)$$

As p_1/p_0 tends to unity then V_R tends to $V_R^0 = al/R_F$. In fact, the flow rate V is measured at room temperature and should be corrected to the column temperature.

Since the value of R_F is not dependent on p_0 it is not possible by reducing p_0 to reduce the temperature of operation of the column. Further, since the viscosity of the gas is independent of the absolute pressure, when p_0 is low, the ratio of the pressures at the two ends of the column becomes excessive if a reasonable rate of flow is to be maintained. Under normal conditions it is convenient for p_0 to be atmospheric pressure. It might, however, be preferable to increase p_0 by a factor of approximately 10 if a very long column is to be used in order to effect a difficult separation. This is due to the fact that the absolute pressure drop from one end of the column to the other for a given retention volume will be independent of p_0; consequently it will be possible to work at a low ratio of p_1/p_0 even with a very long column.

Test of general theory

Test of relationship between pressure, flow rate and retention volume. An experimental test of the theory given above was carried out by the procedure which is described later in the paper. A 4 in. silicic-stearic acid column was set up and a series of separations of acetic, propionic, *n*- and *iso*-butyric acids carried out at four different pressures. The flow rates were measured in each case and from the graphs the retention volume of each acid band was measured.

The theoretical ratio of flow rates is given by (1):

$$\frac{F_1}{F_2} = \frac{(p_1/p_0)^2 - 1}{(p_2/p_0)^2 - 1}.$$

In Table 1 the theoretical ratios of the flow rates are compared with those experimentally determined; agreement is within expected error.

Table 1. *Comparison of calculated with experimentally determined flow rates in a gas–liquid partition chromatogram operated at different pressure ratios*

(The rates in runs *a–d* (Table 2) are compared. For basis of calculation see text.)

	Flow-rate ratios	
	Calc.	Found
a/b	1·44	1·46
a/c	1·92	1·96
a/d	3·36	3·21

From Eqn. 3 it can be seen that as the ratio of the pressures at the two ends of the column tends to unity the factor $\frac{(p_1/p_0)^3 - 1}{(p_1/p_0)^2 - 1}$ tends to 3/2. At this limiting pressure ratio the retention volume of the bands tend to a limit V_R^0. In Fig. 1 the values of

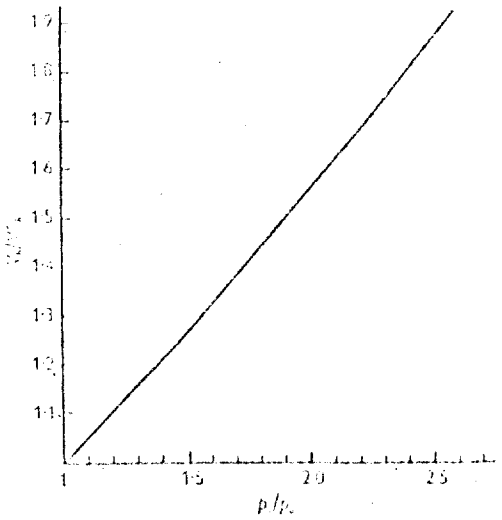


Fig. 1. The relationship between the pressure ratio across the column and the ratio between the observed retention volume and the retention volume at zero pressure difference across the column.

p_1/p_0 are plotted against the value of the ratio V_R/V_R^0; by means of this graph the experimental value of V_R can be corrected to V_R^0. In Table 2 these values of V_R^0 are shown for four experiments carried out at different values of p_1/p_0. It can be

Table 2. *Correction of retention volumes to their limiting values (V_R^0) for runs at different pressure ratios*

Run	Pressure of N_2 applied to column (cm. Hg)	p_1 (N_2 pressure plus atmospheric pressure, p_0, in cm. Hg)	p_1/p_0	V_R^0 (ml.) Acetic acid	Propionic acid	*iso*Butyric acid	*n*-Butyric acid
a	76·0	150·91	2·014	203	466	758	952
b	57·1	132·0	1·762	193	457	749	932
c	45·7	120·61	1·647	194	455	740	922
d	28·9	103·81	1·385	193	464	763	954

seen that the figures agree within 5%, a fairly satisfactory confirmation of the theory, since the assumption is made that the column is uniformly packed.

Derivation of column efficiency. Two methods of calculating column efficiency from the experimental curves are given in order to allow comparison with one another and with data from liquid-liquid columns.

Method 1

Let there be r plates in the column.

Assume that all the material (M equivalents) is put on in the first plate. Then the quantity occurring in the last plate at the maximum concentration is $M/\sqrt{(2\pi r)}$ equivalents (see Martin & Synge, 1941).

Let q be the fraction of the original quantity occurring in any plate, and let the time taken for the centre of the zone to reach the last plate be t min.; then the time taken for contents of last plate to escape $= t/r$ min.

Then $Mq_{max.} = M/\sqrt{(2\pi r)}$ equiv. (cm. and $Mq_{max.}/t/r$ = max. rate of escape = max. slope of the experimental curve = S equiv./min.

Then $S = Mr/t\sqrt{(2\pi r)}$ equiv./min.

Therefore $r = 2\pi S^2t^2/M^2$.

Thus by measuring the maximum slope of the effluent concentration curve, the time to arrive at the maximum slope and the total amount of the material in the zone, the plate number of the column and hence the height equivalent to a theoretical plate (H.E.T.P.) can be obtained. H.E.T.P. is defined for the chromatogram by Martin & Synge (1941).

Method 2

The area between ordinates one standard deviation on either sides of the peak of an error curve is 68·2% of the total. From the theory given by Martin & Synge (1941) the shape of the chromatographic zone should approximate to an error curve and 68·2% of the material should be included between $t/r \pm t/\sqrt{r}$, where r is the total plate number.

The ratio of time taken for the centre of the zone to emerge (t) to the time taken for the middle 68·3% of the zone (τ) is equal to $r/2\sqrt{r}$.

Hence $r = 4t^2/\tau^2$.

A comparison of figures obtained by the two methods for the same column gives fair agreement (Table 3), the agreement increasing with the plate number.

Table 3. *Comparison of column efficiencies obtained by two independent methods*

(For methods 1 and 2 see text.)

Flow rate (ml./min.)	Plate number calculated by Method 1	Method 2
35	550	490
18·2	718	699
10·3	765	730

FACTORS INFLUENCING THE DEGREE OF SEPARATION

Column efficiency

Since diffusion in the gas is so much more rapid than in the liquid, diffusion in the liquid phase will normally be the factor limiting efficiency. At very slow rates of flow lengthways diffusion in the gas along the tube becomes relatively important, and then the efficiency is no longer increased by reducing the rate of flow. Table 4 shows the change of efficiency with flow rates above this limiting value.

Table 4. *Variation of column efficiency with flow rate*

Flow rate (ml./min.)	Efficiency (calc. by method 1) (no. of plates)
35	580
24	540
18·2	720
10	740

The H.E.T.P. shows slight variations from column to column due to differences in the uniformity of packing and of dispersion of the liquid phase. The plate number of any column is proportional to its length, the 4 ft. columns described later having plate numbers varying from 700 to 1200 plates and an 11 ft. column had a plate number of 2000, which might have been greater had it not been for the increased amount of dead space at the bends necessitated by the method of construction.

When diffusion in the liquid phase is the limiting factor, the H.E.T.P. is inversely proportional to the diffusion constant and hence an increase in temperature will lead to an increase in efficiency as the diffusion constant increases. An experimental test showed an efficiency of 265 plates at 65° and an

efficiency of 720 plates at 100° with the same column. This would suggest that in many cases the efficiency of liquid-liquid partition columns could be increased by raising the temperature (cf. Mayer & Tompkins, 1947).

Some variation of efficiency with the nature of the liquid phase will occur because the diffusion constant of the solute in the liquid phase will usually be inversely proportional to the viscosity of the liquid; hence fluids of low viscosity should in general be used.

Drake (1949) has shown that increasing the diameter of a chromatographic column leads to some loss of uniformity of front, and his results could be roughly covered by the assumption that the front made a given angle to the axis of the column irrespective of the column diameter. Since, however, with the gas-liquid column the ratio of length to diameter is very large, moderate increases in diameter should be without measurable effect on efficiency; in fact one 1 ft. column of diameter 1·2 cm. had an efficiency of 850 plates, while with those of 4 mm. diameter the efficiency ranged from 700 to 1200 plates (at 100°).

Change of R_F

Inasmuch as in a homologous series the higher members will have larger latent heats of vaporization, their change of vapour pressure with temperature will be larger than with the lower members, so that the factor of separation will decrease as the temperature increases. This point is demonstrated in Table 5.

Table 5. *Variation of relative R_F values with temperature*

Ratio of retention volumes	Temperature of column		
	65°	100	137
V_R propionic / V_R acetic	2·9	2·3	2·1
V_R *n*-butyric / V_R propionic	2·5	2·0	1·8
V_R *n* butyric / V_R *i*-butyric	1·34	1·27	1·20

Relative R_F values can be altered by changing the nature of the liquid phase. For example, replacement of the stearic acid in the column described later by oleic acid causes a disappearance of the *n*-butyric-*i*-valeric and the *n*-valeric-methylpropionic-*n*-butyric acid separations without materially impairing the separation of *n*- and *iso*-acids. Incorporation of orthophosphoric or pyrophosphoric acids in the liquid phase of the silicone-stearic acid column has a similar effect, but also decreases all R_F values, at the same time decreasing the ratio between them. This effect can no doubt be attributed to an increase in internal pressure of the liquid phase; a similar but more pronounced effect has been noticed in the separation of amines in the comparison of columns with liquid phases of hexadecanol or glycerol. In this case the differences in pressure is so great that an inversion of order with length of chain is observable (James, Martin & Smith, 1952).

APPARATUS

Preparation of the column

Kieselguhr (Celite 545, Johns Manville Co. Ltd., obtainable from Imperial Chemical Industries Ltd.) is size graded by repeated suspension in water in a Fischer 15 cm. High-speed rejection of all the fine and coarse material is carried out in less than 3 min. The washed material is then heated in a muffle for 3 hr. at 500° and then washed with conc. HCl to remove iron and base impurities. After repeated washing with water to remove acid the kieselguhr is oven dried at 115°. The liquid phase consisting of DC550 silicone (Albright & Wilson Ltd., Oldbury, Birmingham), containing 10% (w/w) stearic acid, is added to the kieselguhr (0·5 g. of liquid phase to 1 g. kieselguhr) and the mixture is well stirred.

4 ft. column. A 5 ft. length of 4 mm. internal diameter glass tubes is sealed on one end to form a short thick-walled capillary (*C*, Fig. 2). A small wad of 'Fibreglass'

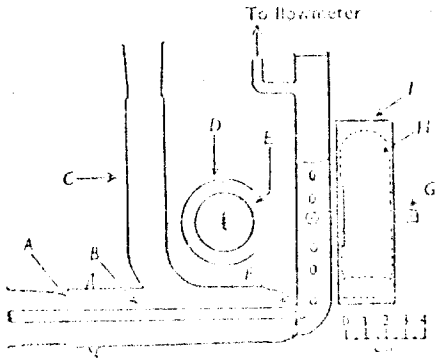


Fig. 2. Side view of titration cell unit. *A*, vapour jacket; *B*, thermocouple; *C*, cell condensation stock; *D*, thermometer; *E*, lamp; *F*, rubber protection gaiters; *G*, quartz bottom to thermometer; *H*, photoelectric cell; *I*, photoelectric cell housing with adjustable aperture.

yarn (S.F.H. 41 obtainable from Filbey Ltd., Piccadilly, London) is packed down the tube up to the capillary to prevent it being blocked by the kieselguhr. A small funnel is attached to the stem by a short length of pressure tubing and is then half-filled with the liquid, the mixture with the column approximately vertical. It is important that the rotating shaft of an Hetheinotor (1950) revolving-cell drive should not be taken from it. When all the kieselguhr has run into the column, the tube is pressed down tightly, and this is secured by a 'Hetheinotor' clamper attached to a length of steel tube, the pressure being applied. The 24 ft. of the column is

packed and a second wad of 'Fibreglass' is pushed down on to the packing. A short piece of cycle-valve tubing is pushed on to the capillary end of the tube and acts as a gasket to prevent leakage from the titration cell into the vapour jacket.

11 ft. column. This column is constructed of three lengths of packed tubing joined by hairpin bends of thick-walled capillary. In this way the whole column can be accommodated within the same vapour jacket as the 4 ft. column. This principle can be used for very long columns if a wide vapour jacket is used.

the bubble past the graduations is timed with a stop-watch. An error of less than 1% is possible over a wide range of flow rates.

The manostat by-passes to the atmosphere any excess gas coming from the nitrogen cylinder through a filter paper fixed with sealing wax to the bottom of the vent tube, when the mercury level is depressed beneath it. The pressure at which this occurs can be varied by adjustment of the level of the mercury reservoir. 'Hunting' is prevented by tightening the screwclip until critical damping occurs.

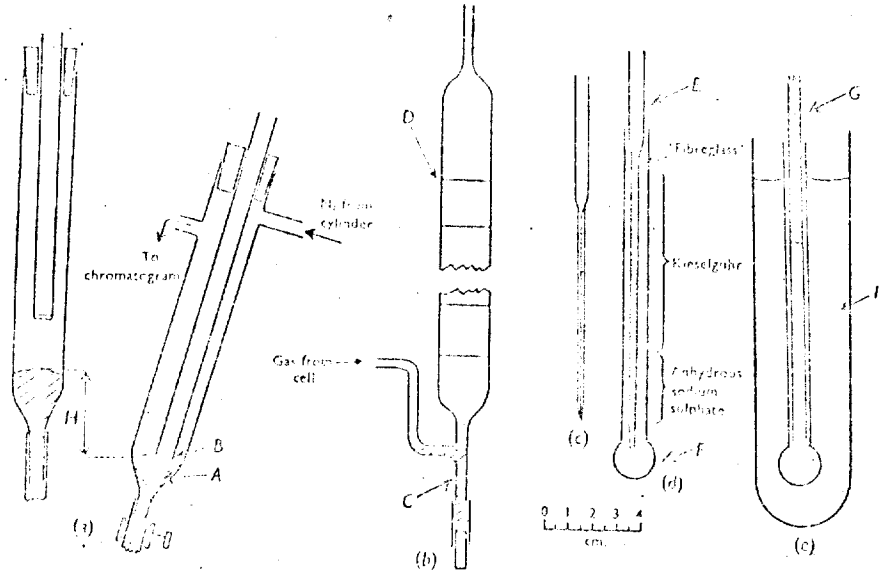


Fig. 3. (*a*) Manostat. *A*, mercury; *B*, vent tube fitted with filter-paper disk; *H*, effective nitrogen head. (*b*) Flowmeter. *C*, ammonium stearate solution; *D*, 10 ml. graduations. (*c*) Micropipette. (*d*) Extraction of free fatty acids into ether. *E*, partition column; *F*, test tube. (*e*) Displacement of fatty acids on to a gas-phase column. *G*, gas-phase chromatogram tube; *I*, heating bath.

Vapour jacket and titration cell

The vapour jacket, a horizontal 1 ft. 1 in. diam. glass tube with a ground-glass joint fitting (the titration-cell unit (Fig. 2), is attached at the other end to the boiler by a ground-glass joint on a side arm. The chromatogram is supported in the jacket by a rubber bung and in the titration cell unit by the valve-tubing gasket (Fig. 2). The gasket, being rubber, limits the choice of liquid in the boiler: methanol (b.p. 65·4°), water (b.p. 100°), ethylene glycol monoethyl ether ('cellosolve') (b.p. 137°) and ethylene glycol (b.p. 200°) are suitable liquids. An air condenser is used for the high-boiling liquids. Phenol red was found to be the most suitable indicator in the titration cell for both visual (0·001%) and automatic (0·01% (w/v) aqueous solution) control of the titration.

Flowmeter and Burette (Figs. 3*b*, *c*)

These are both of conventional design.

A strong, buffered aqueous solution of [illegible] is used in the flowmeter, and in the rubber tube joining the solution to the titration cell [illegible] is found. The [illegible]

Recording burette (Fig. 4)

The burette consists of a horizontal glass tube with a 'Teleothem' (Telegraph Construction and Maintenance Co. Ltd., Tolson Works, Greenwich, London, S.E. 10) gland (*I*) at one end through which passes an 18/25 in. diam. stainless steel rod (*E*). A swelling on the burette tube where it passes through the end plate of the aspirator is insulated with a short length of rubber tube and is held in position by a small clamp (*D*), in this way providing a firm, but slightly flexible, attachment. A similar device allows pressure to be applied to the polythene end without damaging the tube. The other end of the burette is connected to two valves (*A*) consisting of a short length of pressure tubing whose bore is blocked by a short plug of glass rod; the valves may be opened by tightening a screwclip on to the plug. These valves have been found to be more reliable than [illegible] One valve leads to a reservoir filled with a gravel [illegible] solution, and the other to the titration cell through a length of capillary tube.

The [illegible] of Fig. [illegible] is [illegible] of 0·5 in. [illegible] A lead screw (*G*), 1 in. pitch, [illegible] through the [illegible]

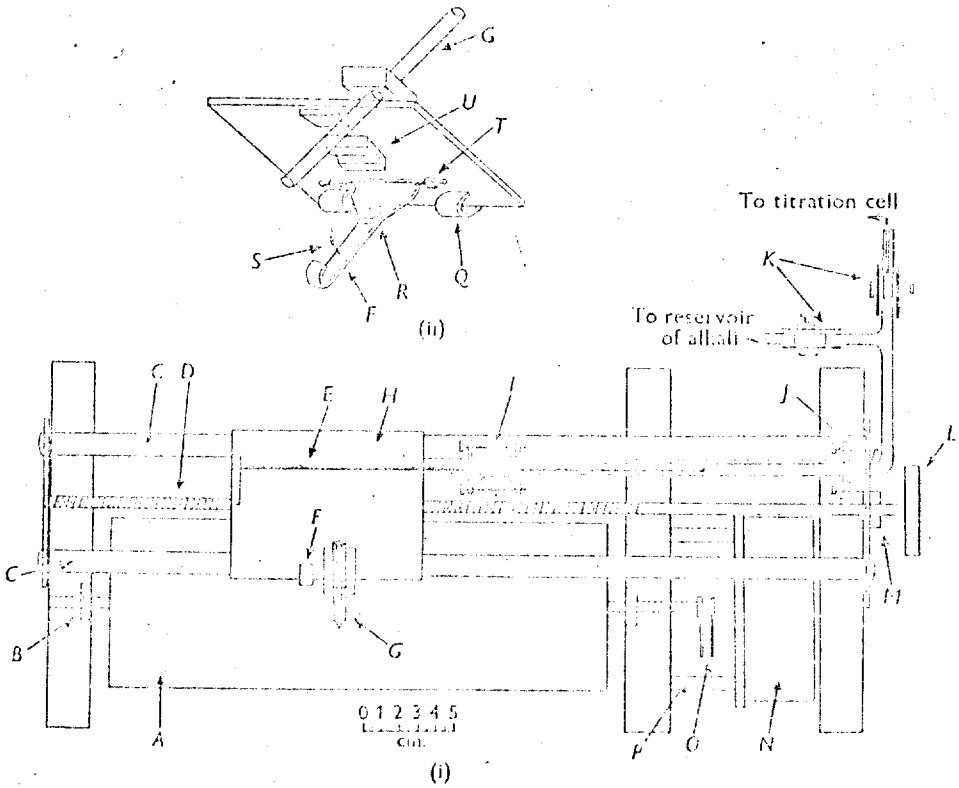


Fig. 4. (i) Plan view of automatic burette: *A*, rotating drum; *B*, drum-shaft bearing; *C*, steel rods; *D*, lead screw; *E*, stainless-steel rod; *F*, arm to disengage lead screw; *G*, pen; *H*, carriage; *I*, "Teflon" plug; *J*, bent-tube clamp; *K*, valves; *L*, pulley; *M*, lead screw bearing; *N*, electric clock movement; *O*, drum drive coupling (only one fork is shown); *P*, clock spacers. (ii) Underside view of carriage (flat springs holding front runner against rod are not shown): *T*, arm bearing half nut; *U*, pen holder; *Q*, carriage runners; *R*, half nut; *S*, spring to hold half nut against lead screw (shown disengaged from the carriage); *T*, pivots of half-nut support; *U*, "Teflon" (Polaud Ltd., Perry Barr, Birmingham) support for lead screw.

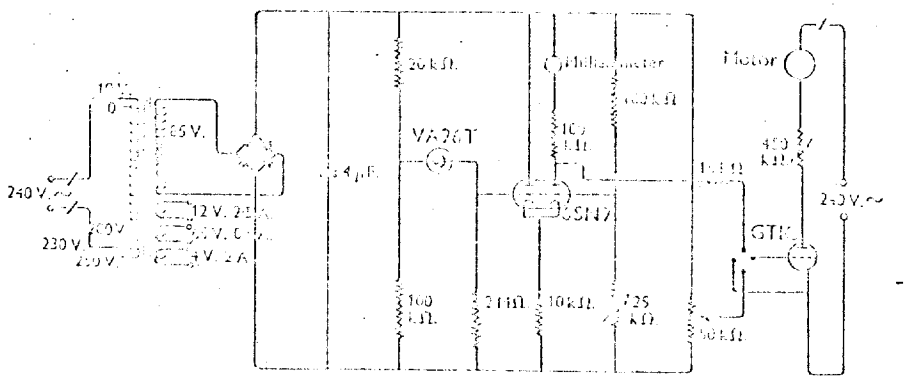


Fig. 5. Circuit of photoelectric control unit.

bars engages with a half nut on a plate pivoted at the back of the carriage (*F*, ii). By the depression of this plate against the action of a spring (*S*, ii) the half nut can be disengaged from the screw to allow rapid filling of the burette. A drum rotates on an axle driven by an electric-clock movement (*N*, Movement 55558/55, 10W. rating, English Clock Systems Ltd., 179 Great Portland Street, London, W. 1) parallel to the lead screw, and the position of the carriage at any time is recorded by a ball-point pen (*G*). The pen is held in a weighted tube (22 g.) which slides freely in a tube (*G*, ii) attached to the carriage, which makes an angle of 70° to the tangent at the point of contact with the drum. Round the drum, whose circumference is 30 cm., a piece of centimetre graph paper is retained by means of two elastic bands of 0·5 in. in diameter which can be readily pulled off the paper on the ends of the drum in order to change the paper. The drum can be rotated either once per hr. or once per 12 hr. by adjusting the position of a pin on a crank at the end of the drum shaft to engage either with a fork on the minute shaft of the clock (*O*) or with another fork on the hour shaft which rotates outside the minute shaft. In the diagram only the inner fork is shown.

The recording burette can be used manually by a handle on the lead screw. It is a very great convenience to use such apparatus instead of other types of burette and a stop-watch. Where the apparatus is expected to be in constant commission the photoelectric control unit will quickly repay the labour involved in its construction.

Photoelectric control unit

The circuit used is shown in Fig. 5. The tube CV1C is a cold-cathode triode. The photoelectric cell (V.A. 26 F Cinema-Television Ltd.) is predominantly green sensitive and consequently no filter is necessary when used with phenolsulphonephthalein as indicator. The photoelectric cell is provided with an adjustable window so that the area of the titration cell scanned can be varied. The lamp used to illuminate the cell is a 12V. (29W.) car bulb. The lamp and photocell are appropriately housed so that a minimum of daylight falls on to the cell, but so that visual observation of the titration is possible. The motor driving the lead screw is fitted with a reduction gear so that the maximum speed of the lead screw is of the order 30 rev./min.; a series choke is included in the motor circuit so that critical damping of the titration can be obtained.

APPLICATION TO THE SEPARATION OF THE VOLATILE FATTY ACIDS

Experimental procedure

The chromatogram supported in the vapour jacket is maintained at a temperature such that the saturated vapour pressures of the acids to be separated lie between 10 and 100 mm. Hg. The temperature ranges from 100° (boiling water) for acids from formic to valeric acid and 137° (boiling cellosolve) for acids from valeric to dodecanoic acid. The solutions of acids free from water is sodium hydroxide are micropipetted into the Tabaqué's plug in contact with the column packing, the N_2 supply from the regulator is attached, the electrical circuits switched on, the recording drum is started, and finally, if necessary, phenolsulphonephthalein solution is added to the titration cell. In many instances of this work additional chlorine is provided by a stream of N_2 through a stainless steel capillary tube. The photoelectric control unit is then switched on. The acids emerge in the gas phase and are absorbed in the water in the titration cell where they are continuously titrated, and simultaneously the titration curve is automatically plotted. The curve obtained is the integral of the conventional chromatogram peak (cf. Moyle, Baldwin & Scarisbrick, 1949); the height of each step gives directly the amount of acid, each cm. corresponding to 3 μg. equiv. throughout these experiments, 0·033N-NaOH being used in the burette. This method of detection of the acids by titration in an aqueous medium limits the chain length of the acids that can be separated; with acids of chain length greater than C_{12} the column would have to be run at a temperature of 150–200° in order to obtain a reasonable retention volume. The maximum possible temperature of the titration cell is 100° (pure water) and with a temperature drop of 50–100° the acids will condense in the capillary at the end of the column and can be prevented from reaching the titration liquid. Titration in non-aqueous media has not in our experience proved satisfactory.

RESULTS

The theory given above assumes that the partial pressures of substances to be separated are negligible; this may not always be so in practice. When they are not negligible the shapes of the zones are distorted because the velocity of gas is appreciably higher in the regions where the partial pressure is high, and this results in regions of high concentration tending to overtake regions of low concentration so that the zone will have a sharp front and a correspondingly longer tail. However, in all the examples quoted below, the partial vapour pressure (estimated from the maximum slope of the recorded step) can be neglected as it does not exceed 5 mm. Hg.

When the solutes obey Henry's law the partition coefficient between the two phases will be independent of concentration and the titration curve obtained will have a symmetrical sigmoid shape. Complete resolution of two zones will result in a plateau between the two curves. Any deviation from Henry's law will cause a deviation, of step shape, from the expected sigmoid curve.

Curve A (Fig. 6) shows the curve obtained in an attempted separation of propionic, *iso*butyric and *n*-butyric, and *iso*- and *n*-valeric acids on a column at 100°, the liquid phase the high boiling point silicone DC 550. The breaks in the curve indicate the ends of the different zones. The extended front and sharp tail of the zones of the higher acids is presumably due to dimerization in the liquid phase causing regions of low acid concentration (relatively high monomer to dimer ratio) to move more rapidly down the column than regions of high acid concentration (relatively low monomer to dimer ratio). This is because the partial pressure of the acid in the vapour is nearly proportional to the concentration of the monomer in the liquid phase.

Incorporation of a long-chain fatty acid of low vapour pressure in the liquid phase will decrease the concentration of monomer by association to such a value that the deviation from ideal zone shape caused by dimerization will be much less. Curve *B* (Fig. 6) shows the result obtained when stearic acid (10% w/w) is incorporated in the silicone liquid phase. The acids are now completely resolved and the *iso*valeric acid zone is shown to consist of two components, the second of which proved to be α-methylbutyric acid ('active' valeric acid). This result was obtained with all commercial samples of 'isovaleric' acid tested (cf. Conrad & Bischoff, 1880; Kahlbaum, 1901). The increased retention volumes are a measure of the effectiveness of the volatile acids as acid association and indicate a diminution of self-dimerization. Most commercial samples of stearic acid contain appreciable amounts of the lower homologues so that a freshly-prepared column loses acid continuously to the titration cell. In so far as the rate of loss of acid is constant, it does not give rise to error in the estimation, but the step has to be measured against a sloping baseline. By running the column at a temperature 50° higher than the normal working temperature for a few hours the volatile impurities can be effectively removed. With pure stearic acid the loss of acid is very small, and in addition the liquid in the titration cell rapidly becomes saturated and thereafter no further titration of the stearic acid entering the cell occurs.

The lower curve (*A*) of Fig. 7 shows the separation of acetic, propionic, *iso*butyric and *n*-butyric acids on a shorter silicone stearic acid column in a smaller flow rate. The upper curve (*B*) is obtained by graphical differentiation of the lower curve and shows a change of zone shape along the series. It is clear that the zones still deviate from the theoretical shape. The acetic acid and propionic acid zones have sharper fronts than tails, the *iso*butyric acid zone is almost symmetrical and the *n*-butyric acid zone has a sharper tail than front. The sharp fronts of the acetic and propionic acid zones are to be attributed to adsorption on the lower column surfaces when the acids were run on a column containing no liquid phase.

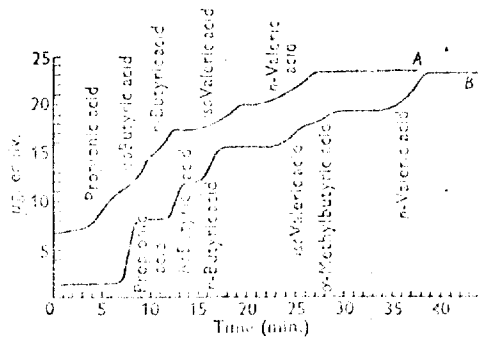


Fig. 6. The separation of propionic, *iso*butyric, *n*-butyric, commercial 'isovaleric' and *n*-valeric acids. *A*, column length, 4 ft.; liquid phase, DC550 silicone; nitrogen pressure, 30 cm. Hg; flow rate 25 ml./min.; column temp., 160°. Incomplete resolution of all the acids. *B*, column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 16 cm. Hg; flow rate, 15 ml./min.; column temp., 160°. The 'isovaleric' band has been almost completely resolved into two components.

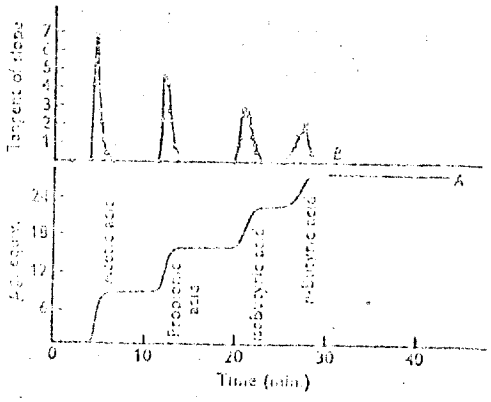


Fig. 7. The separation of acetic, propionic, *iso*butyric and *n*-butyric acids. Column length, 4 ft.; liquid phase, stearic acid (10%, w/w) in DC550 silicone; nitrogen pressure, 45 cm. Hg; flow rate, 33 c.c./min.; column temp., 160°. *A*, experimental curve. *B*, differential of experimental curve.

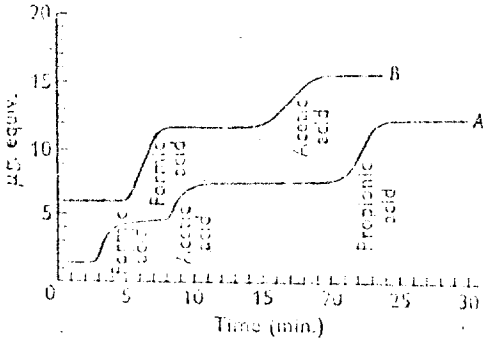


Fig. 8. *A*, the separation of formic, acetic and propionic acids showing tailing of formic acid band. Column length, 4 ft.; liquid phase, stearic acid (10%, w/w) in DC550 silicone; nitrogen pressure, 25 cm. Hg; flow rate, 15 ml./min.; temp. 160°. *B*, the separation of formic and acetic acids, showing nearly symmetrical formic acid band. Column length, 4 ft.; liquid phase, stearic acid (10%, w/w) with phosphoric acid (10%, w/w) in DC550 silicone; nitrogen pressure, 70 cm. Hg; flow rate, 50 ml./min.; temp., 160°.

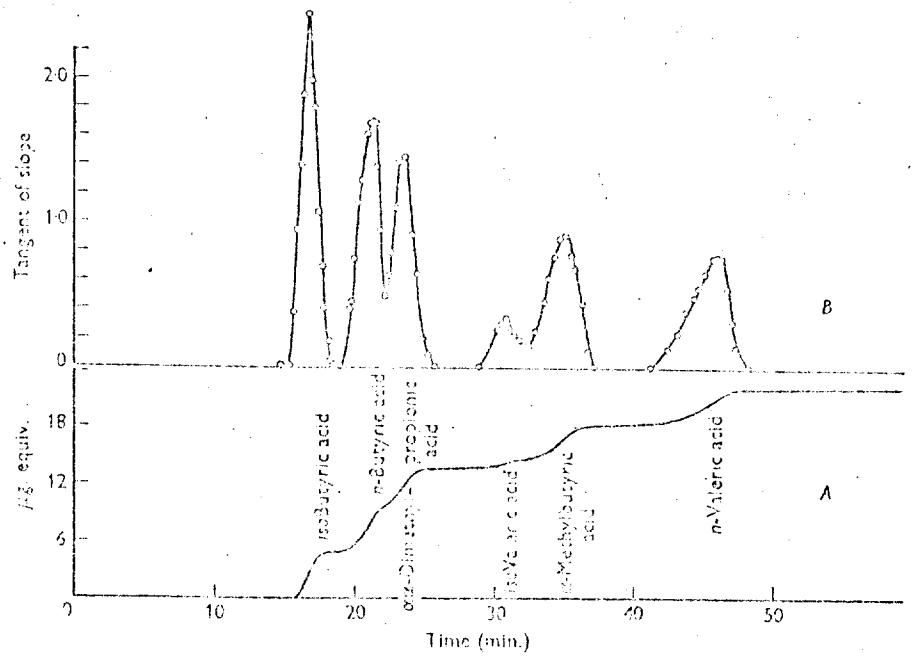


Fig. 9. The separation of the isomers of valeric acid from *n*-butyric and *i*-butyric acids, showing incomplete resolution of α-dimethylpropionic and *n*-butyric acids and of *iso*valeric and α-methylbutyric acids. *A*, experimental curve. *B*, differential of experimental curve. Column length, 14 ft.; liquid phase, stearic acid (10%, w/w) in DC550 silicone; nitrogen pressure, 44 cm. Hg; flow rate, 43 ml./min.; temp., 109°.

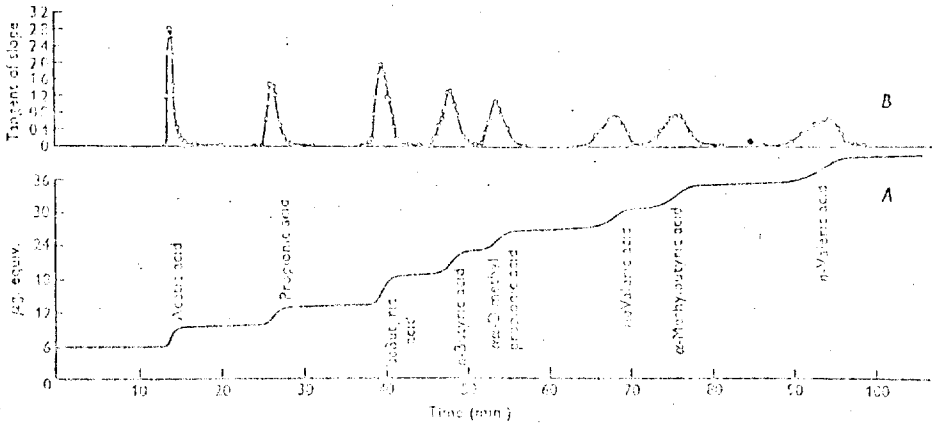


Fig. 10. The separation of acetic, propionic, *n*-butyric and *i*-butyric acids and the isomers of valeric acid, showing the complete resolution of all but [illegible], and [illegible] in head space in [illegible] the series. *A*, experimental curve. *B*, differential of experimental curve. Column length, 11 ft.; liquid phase, stearic acid (10%, w/w) in DC550 silicone; nitrogen pressure, 74 cm. Hg; flow rate, 18·2 ml./min.; temp., 137°.

they showed a non-linear adsorption isotherm with the tail of the acetic zone delayed by some 50 ml. at 100°. This delay increased with the chain length of the fatty acid. In the case of acetic and propionic acids the distortion caused by the adsorption is sufficient to mask completely the effect of the dimerization which has doubtless occurred. With the *iso*butyric acid the two effects are in approximate balance and a nearly symmetrical zone results; with *n*-butyric and higher acids the dominant effect is that of dimerization. Increasing the temperature appears to have a greater effect on dimerization than adsorption, since the symmetrical zone is then given by an acid higher than *iso*butyric acid (see also Fig. 10).

a 4 ft. silicone stearic acid column at 100°. The upper differential curve (obtained as described earlier) shows that complete resolution of *n*-butyric and αα-dimethylpropionic acids and *iso*valeric and α-methylbutyric acids is not attained. To improve this separation a longer column was constructed. Curves *A* and *B* (Fig. 10) show the separation of acetic, propionic, *iso*butyric, *n*-butyric, αα-dimethylpropionic, *iso*valeric, α-methylbutyric and *n*-valeric acids on an 11 ft. silicone stearic acid column at 137°. Complete resolution of all the acids is obtained. The marked change in zone shape with molecular weight is well shown in this series.

Fig. 11 shows the separation of acetic, propionic, *iso*butyric, *n*-butyric, commercial '*iso*valeric', γ-

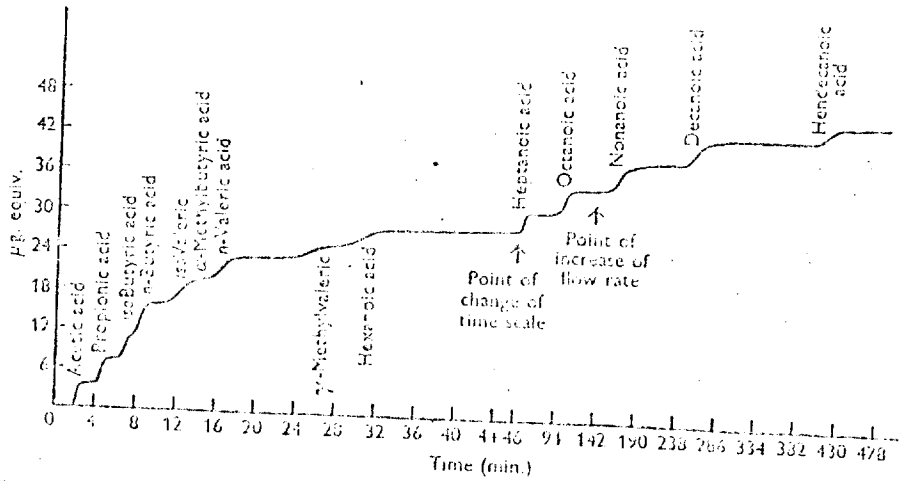


Fig. 11. The separation of acetic, propionic, *n*-butyric, *iso*butyric, *n*-valeric, commercial '*iso*valeric', γ-methylvaleric, hexanoic, heptanoic, octanoic, nonanoic, decanoic and hendecanoic acids. Column length, 11 ft.; liquid phase, stearic acid (10% w/w) in DC 550 silicone; nitrogen pressure, 47 cm. Hg; flow rate, 18·2 ml./min.; temp., 137°. At the time indicated by the first arrow the time scale was changed to 24 min./cm. At the time indicated by the second arrow the pressure was increased to 76·5 cm. Hg and the flow rate to 35 ml./min.

Curve *A* (Fig. 8) shows the separation of formic, acetic and propionic acids on a silicone-stearic acid column. The formic acid zone shows marked tailing and extends backwards into the acetic acid zone so that complete resolution is not obtained. Curve *B* shows the separation of formic and acetic acids when orthophosphoric acid is incorporated in the column liquid phase. The shape of the formic acid zone now approximates to that of the acetic acid zone and indicates that the phosphoric acid has effectively suppressed adsorption. The greater retention volume for each zone also indicates greater dissolution in the liquid phase. A similar effect on tailing is produced by washing the kieselguhr with dilute aqueous phosphoric acid (1% v/v) before the final drying.

Curve *A* (Fig. 9) shows the separation of *iso*butyric, *n*-butyric, αα-dimethylpropionic, *iso*valeric, α-methylbutyric and *n*-valeric acids on

valeric, γ-methylvaleric ('*iso*caproic'), hexanoic, heptanoic, octanoic, nonanoic, decanoic and hendecanoic acids. Dodecanoic acid is not shown as it takes a longer time to emerge than can conveniently be shown on the graph (317 min.). The rate of flow is too high for complete resolution of the lower acids, but it can be seen that a rough quantitative analysis of *n*- and *iso*-acids from C_2 to C_6 can be carried out in 30 min.

All the columns used are highly reproducible; V_R even at different rates of flow varies by less than 5% for a given acid (Table 2) and even the most difficultly separable acids with less than five carbon atoms, namely, αα-dimethylpropionic and *n*-butyric acids, have retention volumes differing by 15% (Table 6). It is thus possible to identify any of these acids by retention volume alone. No marked fall in efficiency with repeated use takes place, the factor

limiting the life being breakage and not deterioration. Recoveries are quantitative within the error of the titration and no indication of incomplete absorption in the titration cell has been found. We have not striven to reduce the errors below a few per cent, though we are of the opinion that the method is essentially a highly accurate one.

Table 6. *Retention volumes of the fatty acids relative to that of n-butyric acid on silicone-stearic acid columns*

Acid	B.p. (°)	Column temp. 100°	Column temp. 137°
Formic	100·7	0·076	—
Acetic	118·1	0·29	0·31
Propionic	141·1	0·37	0·54
isoButyric	154·1	0·77	0·81
n-Butyric	163·5	1·0	1·0
2x-Dimethylpropionic	163·8	1·15	—
isoValeric	176·7	1·51	1·48
x-Methylbutyric	177	1·70	—
nValeric	187	2·17	1·91
x-Methylvaleric	199·1	—	2·94
Hexanoic	205	—	3·53
Heptanoic	223·5	—	6·55
Octanoic	237·5	—	12·0
Nonanoic	254	—	22·0
Decanoic	269–70	—	40·5
Hendecanoic	295*	—	72·8
Dodecanoic	223†	—	138·5

* 100 mm. Hg. † 40 mm. Hg.

A steady increase of retention volume with chain length is found to occur with the straight-chain acids, the retention volume increasing by a factor approx. 1·9 for each increase of chain length of one CH_2 group, formic acid showing the maximum deviation. Fig. 12 shows a plot of log V_R^0 against chain length. It can be seen that the curve joining the *iso* acids runs parallel to that joining the normal acids as would be expected.

The maximum permissible loading of the 5 mm. columns is approximately 1 mg. of each of the lower acids, but if the cross-section area of the column be increased the loading can be increased in the same ratio. The minimum amount of an acid detectable (using 0·038 N-base in the burette) is 0·3 μeq. equiv., i.e. 0·02 mg. for acetic acid. As explained earlier, this lower level is determined only by the method of detection used.

The determination of fatty acids occurring as their sodium salts

In most biological experiments the volatile fatty acids are isolated as their sodium salts, and a simple method of obtaining the acids free from water is necessary before they can be applied to the column. The presence of even a small percentage of water in the fatty acid sample is sufficient to upset the separation, presumably owing to azeotrope formation.

The method adopted consists in drawing up a small sample of an aqueous solution of the sodium salts (30 μl. of an approx. 2 N solution) into a micro-pipette (Fig. 3c) that has been pretreated with a silicone (by washing with a 1% (v/v) solution of DC1107 in chloroform followed by heating at 149° for 30 min.), to render it unwetable by water. A small bubble of air is drawn in and an aqueous

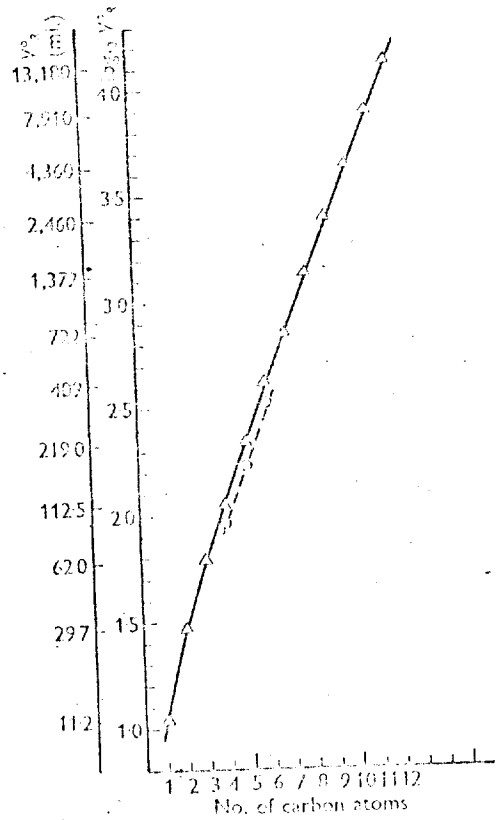


Fig. 12. The relationship between the retention volume at zero pressure difference (V_R^0) and the number of carbon atoms in the lower fatty acids. ○, *n*-Acids; ●, *iso*-acids. Data are for a 4 ft. silicone-stearic acid column at 137° (cf. Fig. 11).

solution of orthophosphoric acid (60% w/v) is drawn up and the two solutions mixed in the wide part of the pipette. The acidified mixture is then run on to the top of a micro-partition column (Fig. 3d), the lower part of which is packed with anhydrous sodium sulphate and the rest with kieselguhr (Celite 545). The aqueous solution of the acids is taken up by the kieselguhr. Ether (0·6 ml.) is then run on to the column to elute the acids and passed through the column under pressure. The eluate is collected in a long narrow test tube

(Fig. 3*d*), whose diameter is slightly greater than that of the chromatogram tube. When all the ether has been collected the chromatogram tube is removed from its vapour jacket and placed in the test tube so that the end of the column is just above the surface of the ether. The test tube is immersed in warm water and air is drawn through the column until all the ether has evaporated. The test tube is then immersed in a heating bath (100° for acids up to valeric, 150° for acids up to dodecanoic acid) and air is drawn through the column for 15 min. The acids move up into the column and condense there. At the end of this time the column is replaced in the vapour jacket, the nitrogen gas turned on and the separation carried out as already described.

SUMMARY

1. The theory of the partition column has been extended to cover a compressible mobile phase. Satisfactory agreement of experiment with theory has been found.

2. Two methods of calculating the height equivalent to a theoretical plate in such columns are derived and compared.

3. Factors influencing the degree of separation are discussed.

4. Gas–liquid partition columns are described for the separation of volatile fatty acids from formic acid to dodecanoic acid.

5. Complete resolution is obtained of all normal acids (and *iso* acids up to at least C_6) on a 4 ft. column and of all the isomers of valeric acid on an 11 ft. column.

6. A method is described for the micro estimation of volatile fatty acids isolated as their sodium salts.

7. A recording fraction is described which may be coupled to a photoelectric control circuit to render the analysis automatic.

We wish to thank Dr R. Searisbrick for a gift of α-methylbutyric acid, Mr E. A. Lipscombe and Mr C. C. F. Payne for the development and construction of the photoelectric control circuit, Mr C. W. Coulson and Mr G. V. Archer for the tracing of the experimental curves, and Mr C. Sutton and co-workers for the photographic copying of all diagrams.

The assistance of Miss A. Moodie and Miss J. Webb with some of the experimental work is gratefully acknowledged.

REFERENCES

Claesson, S. (1946). *Ark. Kemi Min. G. J.* 23A, no. 1.

Conrad, M. & Bischoff, C. A. (1880). *Liebigs Ann.* 204, 113.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* 38, 224.

Dickie, E. (1919). *Analyst. chim. Acta*, 3, 452.

Eberhardt, E., Barker, K. H. & Kitt, G. P. (1949). *Discuss. Faraday Soc.* no. 7, 189.

James, A. T., Martin, A. J. P. & Smith, G. H. (1952). *Biochem. J.* (in the Press).

Ramsbottom, G. W. & Deeds, *Biochem. Soc. J.* 13, 11.

Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* 35, 1358.

Mayer, S. W. & Tompkins, E. R. (1947). *J. American Soc.* 69, 2860.

Moyle, V., Baldwin, E. & Searisbrick, R. (1948). *Biochem. J.* 43, 304.

Phillips, C. S. G. (1949). *Discuss. Faraday Soc.* no. 7, 241.

Turkelteub, N. M. (1940). *J. appl. Chem. U.S.S.R.* 5, 200.

Tucker, N. C. (1943). *Petrol. Refiner*, 22, 95.

MODEL EXPERIMENTS WITH PIGS FOR DETERMINING THE ENERGY UTILIZATION OF ALCOHOL, ACETIC ACID AND LACTIC ACID

W. Jentsch, R. Schiemann and L. Hoffmann (From the Oskar-Kellner Institute of Animal Nutrition in Rostock of the German Academ- of Agricultural Sciences in Berlin, Director: Prof. Dr. habil. W. Laube).

Source: Archiv fur Tierernahrung, Vol. 18, no. 5, pp. 352-37, 1968.

1. Introduction

In the preservation of forage (animal feed) by silo storage, the fermentation products, lactic acid, acetic acid and alcohol are present in such large amounts that they can significantly affect the energy value of silage.

In their proposal of a new system for the energy evaluation of forage and rations, Nehring, Schiemann and Hoffmann (1) have pointed out that the content of fermentation products must be taken into account during the evaluation of silage.

With regard to the energy utilization of fermentation products by monogastric animal species, the short-term tests on 2 pigs for comparing the heat output during the energy-equivalent replacement of 10% cornstarch in the ration by lactic acid, published by Charlet-Lery (4), are known to us from the literature. This author found that starch and lactic acid lead to a heat production of about the same order of magnitude. In the opinion of this author, this result should be collaborated by further tests. We know of no data concerning the energy utilization of acetic acid and alcohol by monogastric animals. The various published short-term tests on man, with measurements of the O_2-consumption after alcohol intake, will not be discussed here since no significant information can be derived from such tests for the purpose of our test formulation.

2. Materials and Methods

Reliable basic information data, needed for estimating fermentation products on the basis of ensilage tests, can be obtained only by carrying out a very large number of tests, since, along with analytical difficulties, the variation in the content of such products in ensilage lies within a range

requiring a large number of ensilage tests in order to determine reliable energy constants of individual fermenation products. For this reason, model experiments were carried out according to the differential process on pigs of the "German pedigree hog" stock, weighing from 140 to 180 kg, to determine the energy utilization of alcohol, lactic and acetic acids and an alcohol-lactic acid mixture (mass ratio 1:1).

In preliminary tests, different possible ways of administering the compounds were checked, since the animals did not absorb (consume) free acids at an appropriate level with the basic animal feed otherwise used by us. Neutralization of the acids with NaOH and/or $Ca(OH)_2$ imposed a great load on mineral metabolism. More than 10 l water was consumed by the animals and the feed was finally turned down (i.e. they refused to eat).

In combination with large amounts of saccharose, 400 g of acid was consumed by the animals; for this reason, a basic feed containing 500 g saccharose was used. In the subsequent total metabolism tests, difficulties were again experienced in administering acetic acid with this amount of cane sugar. The animals accepted 300 g acetic acid only when this amount was combined with further 300 g saccharose. In spite of the sugar addition, the intake of feed extended almost until the following feeding time, since the animals consumed the feed only hesitantly and mostly only after receiving additional drinking water. In view of these difficulties in administration, extensive scattering of the results was to be expected in advance in the case of acetic acid.

The composition and digestibility of the basic animal feed are shown in Table 1.

TABLE 1

Composition and digestibility of the basic feed ration.

Futtermittel in g Tr.-S.		Durchschnittliche Nährstoffzusammensetzung		Verdaulichkeit in %
Gerstenschrot	574	Org. Subst.	95,83%	88,3 ± 0,4
Saccharose	500	Rohprotein	19,07%	85,2
Fischmehl	204	Rohfett	2,39%	83,6
Leinextraktionsschrot	204	Rohfaser	3,80%	20,0
	?	NFE	70,57%	92,9
Mineralstoffmischung	20	Kohlenstoff	44,32%	—
		Bruttoenergie	4373 cal/g	86,2 ± 0,4
		verd. Energie	3718 ± 18 cal/g	
		umsetzb. Energie	3523 ± 17 cal/g	

Mit diesem Grundfutter konnten die in Tab. 2 aufgeführten Zulagehöhen erreicht werden.

1 - Feed components, in g dry substance
2 - Average nutrient composition
3 - Digestibility, in %
4 - Barley grits
5 - Fish meal
6 - Linseed oil cake meal
7 - Mineral mixture
8 - Crude (raw) protein
9 - Raw fat
10 - Raw fiber
11 - Carbon
12 - Gross energy
13 - Digestible energy
14 - Metabolizable energy

With this basic feed, the supplement levels listed in Table 2 could be achieved.

TABLE 2

	in g/Tag	in % der Bruttoenergie des Grundfutters	Zahl der ausgewerteten Versuche
Alkohol	290	31	6
Alkohol (A)	197	30	2+4**
Milchsäure (M)	204		
Milchsäure	403	19	6
Essigsäure	303	16*)	6

*) Bei Essigsäurezulage wurden zusätzlich 300 g Saccharose ≙ 18% der Bruttoenergie des Grundfutters verabreicht

** Diese 4 Versuche wurden in einer späteren Versuchsreihe durchgeführt. Das dazugehörige Grundfutter ist in der Veröffentlichung über die Energieverwertung der Kleien (5) beschrieben. In den Tabellen 2–4 sind die Mittelwerte und Streuungen aller 6 Einzelversuche angeführt, da bei der Wiederholung mit 4 Tieren die ersten Ergebnisse mit 2 Tieren voll bestätigt wurden

1 - In g/day
2 - In % of the gross energy of the basic feed
3 - Number of evaluated tests
4 - Alcohol

*When acetic acid was used as supplement, an additional 300 g saccharose (equal to or more than 18% of the gross energy of the basic feed) was administered.

**These 4 tests were carried out during a later series of tests. The basic feed used in these tests is described in the published article on energy utilization of clover (5). In Tables 2-4, the average values and scattering of all 6 individual tests are listed, since the first results obtained with 2 animals were fully confirmed during the repetition of tests with 4 animals.

Alcohol consumption presented no difficulties in principle. In preliminary experiments, the animals consumed 400 g alcohol daily. With this amount of alcohol, considerable losses due to exhaling and evaporation occurred, and the animals exhibited a completely apathic behavior. For this reason, only one animal was fed this amount of alcohol in the main test, and in other animals the amount was reduced to 300 g. When losses due to exhalation and evaporation are taken into account, an average amount of metabo lized alcohol of 290 g (listed in Table 2) was obtained, corresponding to 31% of the gross energy of the basic feed. Approximately the same supplement level was achieved with the alcohol-lactic acid mixture. For the acids, the energy fraction of the supplement amounted to 16 and 19% of the energy of the basic feed.

In order to exclude as far as possible any evaporation of volatile products (alcohol and acetic acid) during sample weighing, the supplements were weighed from tightly closed containers just prior to feeding and immediately diluted with water. In order to determine the unavoidable losses due to evaporation after the feed administration, about 1% of the air output was pumped with a vacuum pump from the breathing chamber through a potassium bichromate solution and a gas meter. Volatile products were quantitatively absorbed in the potassium bichromate solution.

The volatile alcohol was determined by the modified procedure of Wwissbach and Laube (3). With alcohol, losses occurred due to exhalation and evaporation. No measurable evaporation was noted during the feeding of acetic acid. The evaporation level of alcohol varied greatly according to the amount of alcohol administered and the rate of feed intake (consumption). The highest alcohol evaporation rate of 18.8%, during weighing of the alcohol sample, was noted with a 400 g alcohol weighed sample and during reluctant feed consumption, and the lowest evaporation rate of 1.6% was noted during the mixing of 200 g alcohol and 200 g lactic acid. The average alcohol evaporation observed during the period of alcohol supplement was 10% (in individual animals 2.6 to 18.8%), and 2.8% during the period when the alcohol-lactic acid mixture was given (in individual animals 1.6 to 4.1%).

"Primasprit" (trade name for undenatured absolute alcohol) was used as the alcohol supplement. By density determination, the alcohol content was found to be 94-96%; which corresponds to a C-content of 49.51% and a calorific value of 6752 cal/g. For both acids, the C-content was determined directly

and the energy content was calculated from this value. The aqueous lactic acid solution had a C-content of 32.93% and 32.73%, and, accordingly, an energy content of 2979 and 2961 cal/g, while acetic acid had a C-content of 39.37% and an energy content of 3432 cal/g.

3. Results and Discussion

The changes in the digestion of the basic feed, caused by the addition of supplements, are shown in Table 3.

TABLE 3

Changes in the digestion of the basic feed caused by supplements (with an indication of the extent of variations).

Zulage (1)	Rohprotein (2) g/Tag	Rohfett (3) g/Tag	Rohfaser (4) g/Tag	Verdauungsänderungen, in Nettoenergie umgerechnet, in % des Gesamtansatzes (5)
	Alcohol	Lactic acid		
Alkohol	+ 1,7 (− 3,9 bis + 9,3)	+ 1,9 (− 1,1 bis + 4,7)	+ 1,4 (− 9,6 bis + 12,0)	+ 1,5 (− 1,3 bis + 2,6)
Alkohol + Milchsäure	+ 1,4 (− 5,8 bis + 14,9)	+ 4,1 (+ 0,9 bis + 8,0)	− 1,1 (− 10,1 bis + 7,6)	+ 2,3 (− 0,2 bis + 7,3)
Milchsäure	+ 5,2 (− 1,7 bis + 9,9)	+ 5,6 (− 3,9 bis + 8,3)	+ 0 (− 3,1 bis + 3,1)	+ 5,8 (− 2,9 bis + 7,8)
Essigsäure	+ 3,8 (− 11,1 bis + 21,9)	+ 2,9 (− 7,5 bis + 7,9)	+ 4,4 (− 6,9 bis + 12,2)	+ 5,1 (− 6,3 bis + 8,5)
	Acetic acid			

1 - Supplement
2 - Raw protein, g/day
3 - Raw fat, g/day
4 - Raw fiber, g/day
5 - Digestive changes, calculated as not energy, in percent of the total increase.

On the average, directional positive deviations were noted with all nutrient groups, and especially with acid supplements. This is in agreement with the observations of Charlet-Lery (4). No further tests were carried out to explain this secondary finding. It is conceivable that the secretion of digestive enzymes is stimulated by the acid supplemen ts. The expected effect on the energy increase during the supplement addition period, caused by changes in the digestibility of the basic feed, is shown in column 5 of

Table 3. These values were used to correct the directly measured total increase, since changes in digestion and live weight and the different consumption of basic feed are directly related to the energy exchange (metabolism). The same factors as those reported by Schiemann, Hoffmann and Nehring (2) were used for making these corrections.

The energy metabolism of the supplements is shown in Table 4.

Table 4

Zulage (1)	n	(2) Brutto-energie in cal/g reine Zulage	verdaul. (3) Energie in cal/g Zulage	verdaul. (4) Energie in % der Brutto-energie	umsetzb. (5) Energie in cal/g verdaute Zulage	umsetzb. (6) Energie in % der Energie der verd. Zulage	Ansatz in (7) cal/g verd. Zulage	Verwertung (8) in % der umsetzb. Energie
Alkohol	6	7111	7099 ± 87	99,8 ± 3,1	7148 ± 61	100,5 ± 0,8	5164 ± 251	72,2 ± 3,4
Alkohol + Milchsäure	6	5463	5363 ± 66	98,2 ± 1,3	5499 ± 10	100,7 ± 0,2	3960 ± 157	72,0 ± 2,8
Milchsäure	6	3619	3407 ± 41	94,1 ± 1,1	3654 ± 141	100,9 ± 3,9	2731 ± 140	74,8 ± 4,8
Essigsäure	6	3487	3480 ± 100	99,8 ± 3,1	3598 ± 87	100,6 ± 2,5	2100 ± 543	59,9 ± 14,7

1 - Supplement
2 - Gross energy in cal/g of pure supplement
3 - Digestible energy in cal/g of supplement
4 - Digestible energy in percent of gross energy
5 - Metabolizable energy in cal/g of digested supplement
6 - Metabolizable energy in percent of the energy of the diluted supplement
7 - Increase in cal/g of diluted supplement
8 - Utilization in percent of metabolizable energy

From the tabulated data on the digestible energy we can see that the supplements are approximately 100% resorbed. The higher scattering of the mean values of the digestion coefficients, in comparison to the feed supplements, can in part be attributed to the fact that the digestibility of the basic feed was very high, that the feces excretion was very irregular in view of the low thrust, and therefore test periods of 10-13 days duration are somewhat too short. In view of the difficulties experienced in the administration of supplements, the test periods could not be extended.

The metabolizable energy is higher by 0.5-0.9% than the energy of the digestible nutrient. Differences in urine excretion during the basic feed supplement periods show that, during the supplement periods, more nitrogen was used in the required buildup of connective tissue during increased fat deposition. The protein increase (kcal) amounted to 6.3% of the total increase for alcohol, 5.9% for the alcohol-lactic acid mixture, 0.2% for lactic acid, and 4.3% for acetic acid.

The utilization of the metabolizable energy of alcohol and lactic acid corresponds, with 72 and 75%, resp., to the utilization of carbohydrates, as was determined by us in previous tests (2). The two tests with a mixture of alcohol and lactic acid confirm this statement. The results obtained with acetic acid are, as was to be expected, affected by a high degree of scattering.

The average value of 60% found for the utilization of acetic acid is in good agreement with the theoretically expected values. For the activation of acetate 2 ATP-equivalents are additionally required (ATP=adenosine triphosphate), which explains the approximately 20% lower utilization of acetic acid, as compared to lactic acid and alcohol. In spite of the high scattering, the result, therefore, appears to be representative for the estimate of the energy utilization of acetic acid, when difficulties concerning the method are taken into account.

The net energy value of alcohol was determined at 5.16 kcal/g, that of lactic acid at 2.73 kcal/g, and tha of acetic acid at 2.1 kcal/g.

Thus, energy figures for the principal fermentation products present in silage are available. After completion of studies currently under way at our Institute on the routine-like analytical detection of fermentation products, it is planned to submit appropriate proposals for calculating the energy feed value of silage in pigs.

SUMMARY

In model experiments (total metabolism tests) the energy utilization of alcohol, lactic acid and acetic acid in pigs was determined, under conditions of fat production in full-grown animals.

Differential analysis yielded the following results:

	1. Ansatz in cal/g verd. Zulage	Verwertung in % der umsetzbaren Energie 2.
Alcohol		
Alkohol	5164 ± 251	72,2 ± 3,4
Milchsäure	2731 ± 140	74,8 ± 4,8
Essigsäure	2100 ± 543	59,9 ± 14,7
lactic acid		
Acetic acid		

1 - Increase, in cal/g of diluted supplement

2 - Utilization, in percent of metabolizable energy

BIBLIOGRAPHY

1 NEHRING, K., R. SCHIEMANN und L. HOFFMANN: Sitz.-Ber. dtsch. Akad. Landwirtsch.-Wiss. Berlin XV, H. 21, S. 19 (1966)
2 SCHIEMANN, R., L. HOFFMANN und K. NEHRING: Arch. Tierernähr. 11, 265 (1961)
3 WEISSBACH, F., und W. LAUBE: Z. landwirtsch. Vers.- und Unters.-Wesen 10, 65 (1964)
4 CHARLET-LERY, G.: Proceedings of the 3rd Symp. on Energy Metabolism. EAAP-Publ. Nr. 11, 279 (1965)
5 JENTSCH, W., L. HOFFMANN und R. SCHIEMANN: Arch. Tierernähr. 18, 344 (1968)

Eingegangen: 21. Juni 1967

Aus dem Oskar-Kellner-Institut für Tierernährung Rostock
der Deutschen Akademie der Landwirtschaftswissenschaften zu Berlin
(Direktor: Prof. Dr. habil. W. LAUBE)

Arch. Tierernahr 18(5): 352ff.

W. JENTSCH, R. SCHIEMANN und L. HOFFMANN

Modellversuche mit Schweinen zur Bestimmung der energetischen Verwertung von Alkohol, Essig- und Milchsäure

1. Einleitung

Bei der Konservierung der Futterstoffe durch Silierung treten die Gärungsprodukte Milchsäure, Essigsäure und Alkohol in solchen Mengen auf, daß sie den Energiewert der Silagen wesentlich beeinflussen können.

Im Vorschlag eines neuen Systems zur energetischen Bewertung der Futterstoffe und Rationen wurde von NEHRING, SCHIEMANN und HOFFMANN [1] darauf hingewiesen, daß bei der Bewertung der Silagen die Gehalte an Gärungsprodukten zu berücksichtigen sind.

Aus der Literatur sind uns über die Verwertung der Gärungsprodukte durch monogastrische Tierarten die Kurzzeitversuche an 2 Schweinen für den Vergleich der Wärmeproduktion beim energieäquivalenten Ersatz von 10% Maisstärke in der Ration durch Milchsäure von CHARLET-LERY [4] bekannt. Sie kam zu dem Ergebnis, daß Stärke und Milchsäure zu einer etwa gleich hohen Wärmebildung führen. Ihrer Auffassung nach sollte dieses Ergebnis durch weitere Versuche erhärtet werden. – Über die energetische Verwertung der Essigsäure und des Alkohols durch monogastrische Tiere sind uns keine Angaben bekannt. Die verschiedenen publizierten Kurzzeitversuche an Menschen mit Messungen des O_2-Verbrauches nach Alkoholaufnahme sollen hier nicht besprochen werden, da aus ihnen für unsere Versuchsfragestellung keine wesentlichen Hinweise abgeleitet werden können.

2. Material und Methodik

Gesicherte Unterlagen für die Bewertung der einzelnen Gärungsprodukte auf Grund von Versuchen mit Silagen zu erhalten, ist nur mit einem sehr hohen Versuchsaufwand zu erreichen, da neben den analytischen Schwierigkeiten die Variation der Gehalte dieser Produkte in den Silagen sich in solchen Grenzen hält, daß für die Ermittlung gesicherter energetischer Kennzahlen der einzelnen Gärungsprodukte eine große Zahl von Versuchen mit Silagen erforderlich wäre. Es wurden daher mit Schweinen der Rasse Deutsches Edelschwein im Masseabschnitt von etwa 140 bis 180 kg Modellversuche zur Bestimmung der energetischen Verwertung von Alkohol, Milch-, Essigsäure und einer Mischung von Alkohol und Milchsäure im Masseverhältnis 1 : 1 nach dem Differenzverfahren durchgeführt.

In Vorversuchen wurden verschiedene Applikationsmöglichkeiten geprüft, da die Tiere die Säuren in freier Form mit dem von uns sonst verwendeten Grundfutter nicht in entsprechender Höhe aufnahmen. Neutralisation der Säuren mit

NaOH bzw. $Ca(OH)_2$ führte zu starker Belastung des Mineralstoffwechsels. Es wurden mehr als 10 l Wasser von den Tieren aufgenommen und das Futter schließlich verweigert.

In Verbindung mit größeren Mengen Saccharose wurden von den Tieren 400 g Säure aufgenommen. Deshalb wurde ein Grundfutter mit 500 g Saccharoseanteil verwendet. In den anschließenden Gesamtstoffwechselversuchen bereitete die Essigsäureverabreichung mit dieser Rohrzuckermenge erneut Schwierigkeiten. Die Tiere nahmen 300 g Essigsäure nur in Verbindung mit weiteren 300 g Saccharose auf. Trotz des Zuckerzusatzes erstreckte sich die Futteraufnahme nahezu bis zur nächsten Futterzeit, da die Tiere das Futter nur zögernd und meist nur nach erneuter Wasserzugabe aufnahmen. Auf Grund der Applikationsschwierigkeiten war bei der Essigsäure von vornherein mit größeren Streuungen der Ergebnisse zu rechnen.

In Tab. 1 sind Zusammensetzung und Verdaulichkeit des Grundfutters zusammengestellt.

Tabelle 1
Zusammensetzung und Verdaulichkeit der Grundfutterration

Futtermittel in g Tr.-S.		Durchschnittliche Nährstoffzusammensetzung		Verdaulichkeit in %
Gerstenschrot	574	Org. Subst.	95,83%	88,3 ± 0,4
Saccharose	500	Rohprotein	19,07%	85,2
Fischmehl	204	Rohfett	2,39%	83,6
Leinextraktionsschrot	204	Rohfaser	3,80%	20,0
		NFE	70,57%	92,9
Mineralstoffmischung	20	Kohlenstoff	44,32%	–
		Bruttoenergie	4373 cal/g	86,2 ± 0,4
		verd. Energie	3718 ± 18 cal/g	
		umsetzb. Energie	3525 ± 17 cal/g	

Mit diesem Grundfutter konnten die in Tab. 2 aufgeführten Zulagehöhen erreicht werden.

Tabelle 2
Durchschnittliche Höhe der Zulagen und Anzahl der ausgewerteten Versuche

	in g/Tag	in % der Bruttoenergie des Grundfutters	Zahl der ausgewerteten Versuche
Alkohol	290	31	6
Alkohol (A)	197	30	2+4**
Milchsäure (M)	204		
Milchsäure	403	19	6
Essigsäure	303	16*)	6

* Bei Essigsäurezulage wurden zusätzlich 300 g Saccharose ≙ 18% der Bruttoenergie des Grundfutters verabreicht

** Diese 4 Versuche wurden in einer späteren Versuchsreihe durchgeführt. Das dazugehörige Grundfutter ist in der Veröffentlichung über die Energieverwertung der Kleien (5) beschrieben. In den Tabellen 2–4 sind die Mittelwerte und Streuungen aller 6 Einzelversuche angeführt, da bei der Wiederholung mit 4 Tieren die ersten Ergebnisse mit 2 Tieren voll bestätigt wurden

Die Alkoholaufnahme bereitete im Prinzip keine Schwierigkeiten. Die Tiere nahmen in Vorversuchen 400 g Alkohol pro Tag auf. Bei dieser Alkoholmenge traten aber beträchtliche Ausatmungs- und Verdunstungsverluste auf, und die Tiere zeigten ein völlig apathisches Verhalten. Daher wurde diese Alkoholmenge im Hauptversuch nur noch an ein Tier verfüttert und bei den anderen Tieren auf 300 g reduziert. Unter Berücksichtigung der Ausatmungs- und Verdunstungsverluste ergab sich die in Tab. 2 angeführte durchschnittlich umgesetzte Alkoholmenge von 290 g entsprechend 31% der Bruttoenergie des Grundfutters. Etwa gleiche Zulagenhöhe wurde mit der Mischung Alkohol + Milchsäure erreicht. Bei den Säuren betrug der Energieanteil der Zulage 16 bzw. 19% der Bruttoenergie des Grundfutters.

Um bei den flüchtigen Substanzen (Alkohol und Essigsäure) Verdunstungen bei der Einwaage weitestgehend auszuschalten, wurden die Zulagen aus gut verschlossenen Gefäßen kurz vor der Verfütterung eingewogen und sofort mit Wasser verdünnt. Zur Erfassung der unvermeidlichen Verdunstungsverluste nach der Futterverabreichung wurde aus der Respirationskammer mit einer Vakuumpumpe etwa 1% des Luftdurchsatzes durch Kaliumbichromatlösung und eine Gasuhr gesaugt. In der Kaliumbichromatlösung erfolgte eine quantitative Absorption der flüchtigen Substanzen.

Die Bestimmung des flüchtigen Alkohols erfolgte nach der von WEISSBACH und LAUBE [3] modifizierten Vorschrift. Beim Alkohol traten Verdunstungs- und Ausatmungsverluste auf. Bei Essigsäureverfütterung wurde keine meßbare Verdunstung bemerkt. Die Verdunstungshöhe des Alkohols war in Abhängigkeit von der verabreichten Alkoholmenge und der Geschwindigkeit der Futteraufnahme durch die Tiere sehr unterschiedlich. Die größte Alkoholverdunstung von 18,8% der Alkoholeinwaage wurde bei 400 g Alkoholeinwaage und zögernder Futteraufnahme beobachtet, die niedrigste von 1,6% bei der Mischung von 200 g Alkohol und 200 g Milchsäure. Im Durchschnitt betrug die Alkoholverdunstung in der Periode mit Alkoholzulage 10,0% (bei den Einzeltieren 2,6 bis 18,8%) und in der Periode mit der Mischung von Alkohol und Milchsäure im Durchschnitt 2,8% (bei den Einzeltieren 1,6 bis 4,1%).

Als Alkoholzulage wurde „Primasprit"[1] verwendet. Der Alkoholgehalt wurde durch Bestimmung der Dichte mit 94,96% ermittelt. Das entspricht einem C-Gehalt von 49,51% und einem Brennwert von 6752 cal/g. Von den beiden Säuren wurde der C-Gehalt direkt bestimmt und auf Grund dessen der Energiegehalt berechnet. Die wäßrige Milchsäurelösung hatte einen C-Gehalt von 32,93 bzw. 32,73% und dementsprechend einen Energiegehalt von 2979 bzw. 2961 cal/g, die Essigsäure einen C-Gehalt von 39,37% und dementsprechend einen Energiegehalt von 3432 cal/g.

3. Ergebnisse und Diskussion

Die Zulagen bewirkten Verdauungsveränderungen des Grundfutters, die in Tab. 3 zusammengestellt sind.

[1] Handelsname für unvergällten absoluten Alkohol.

Tabelle 3

Durch die Zulagen bedingte Verdauungsänderungen des Grundfutters (mit Angabe der Schwankungsbreite)

Zulage	Rohprotein g/Tag	Rohfett g/Tag	Rohfaser g/Tag	Verdauungsänderungen, in Nettoenergie umgerechnet, in % des Gesamtansatzes
Alkohol	+ 1,7 (− 3,9 bis + 9,3)	+ 1,9 (− 1,1 bis + 4,7)	+ 1,4 (− 9,6 bis + 12,0)	+ 1,5 (− 1,3 bis + 2,6)
Alkohol + Milchsäure	+ 1,4 (− 5,8 bis + 14,9)	+ 4,1 (+ 0,9 bis + 8,0)	− 1,1 (− 10,1 bis + 7,6)	+ 2,3 (− 0,2 bis + 7,3)
Milchsäure	+ 5,2 (− 1,7 bis + 9,9)	+ 5,6 (− 3,9 bis + 8,3)	+ 0 (− 3,1 bis + 3,1)	+ 5,8 (− 2,9 bis + 7,8)
Essigsäure	+ 3,8 (− 11,1 bis + 21,9)	+ 2,9 (− 7,5 bis + 7,9)	+ 4,4 (− 6,9 bis + 12,2)	+ 5,1 (− 6,3 bis + 8,5)

Im Durchschnitt ergaben sich bei allen Nährstoffgruppen gerichtet positive Abweichungen, besonders bei den Säurezulagen. Dies deckt sich mit den Beobachtungen von CHARLET-LERY [4]. Zur Aufklärung dieses Nebenbefundes wurden keine weiteren Versuche unternommen. Man könnte an eine Stimulierung der Sekretion von Verdauungsfermenten durch die Säuregaben denken. In Spalte 5 ist die durch die Verdaulichkeitsänderung des Grundfutters auf den Energieansatz der Zulageperiode zu erwartende Auswirkung aufgenommen. Mit diesen Größen wurde der direkt gemessene Gesamtansatz korrigiert, da die Verdauungsänderungen wie die Lebendmasseveränderungen und der unterschiedliche Grundfutterverzehr in direkter Beziehung zum Energiewechsel stehen. Für die Korrekturen wurden die gleichen Faktoren wie von SCHIEMANN, HOFFMANN und NEHRING [2] verwendet.

In Tab. 4 ist der Energieumsatz der Zulagen dargestellt.

Aus den Angaben über die verdauliche Energie ist zu ersehen, daß die Zulagen annähernd zu 100% resorbiert wurden. Die im Vergleich zu Futterstoffzulagen erhöhten Mittelwertstreuungen der Verdauungskoeffizienten können teilweise darauf zurückgeführt werden, daß die Verdaulichkeit des Grundfutters sehr hoch war, die Kotausscheidungen wegen des geringen Schubs sehr unregelmäßig erfolgten und demzufolge 10–13 Tage dauernde Versuchsperioden etwas zu kurz sind. Wegen der Applikationsschwierigkeiten konnten die Perioden jedoch nicht verlängert werden.

Die umsetzbare Energie liegt um 0,5–0,9% höher als die Energie des verdaulichen Nährstoffes. Aus den Differenzen der Harnausscheidungen zwischen Grundfutter- und Zulageperioden geht hervor, daß in den Zulageperioden mehr N für den notwendigen Aufbau des Bindegewebes bei der vermehrten Fettablagerung angesetzt wurde. Der Proteinansatz (kcal) betrug beim Alkohol 6,3%, bei der Mischung Alkohol + Milchsäure 5,9%, bei Milchsäure 0,2% und bei Essigsäure 4,3% des Gesamtansatzes.

Tabelle 4
Der Energieumsatz der Zulagen

Zulage	n	Brutto-energie in cal/g reine Zulage	verdaul. Energie in cal/g Zulage	verdaul. Energie in % der Brutto-energie	umsetzb. Energie in cal/g verdaute Zulage	umsetzb. Energie in % der verd. Zulage	Ansatz in cal/g verd. Zulage	Verwertung in % der umsetzb. Energie
Alkohol	6	7111	7099 ± 87	99,8 ± 3,1	7148 ± 61	100,5 ± 0,8	5164 ± 251	72,2 ± 3,4
Alkohol + Milchsäure	6	5463	5363 ± 66	98,2 ± 1,3	5499 ± 10	100,7 ± 0,2	3960 ± 157	72,0 ± 2,8
Milchsäure	6	3619	3407 ± 41	94,1 ± 1,1	3654 ± 141	100,9 ± 3,9	2731 ± 140	74,8 ± 4,8
Essigsäure	6	3487	3480 ± 100	99,8 ± 3,1	3508 ± 87	100,6 ± 2,5	2100 ± 543	59,9 ± 14,7

Die Verwertung der umsetzbaren Energie von Alkohol und Milchsäure entspricht mit 72 bzw. 75% der Verwertung der Kohlenhydrate, wie sie von uns [2] in früheren Versuchen ermittelt wurde. Die beiden Versuche mit einem Gemisch von Alkohol und Milchsäure bestätigen diese Aussage. Die Ergebnisse mit Essigsäure sind, wie erwartet wurde, mit hohen Streuungen behaftet.

Der im Mittel gefundene Wert von 60% für die Verwertung der Essigsäure steht mit den theoretischen Erwartungen in guter Übereinstimmung. Für die Aktivierung des Azetats werden zusätzlich 2 ATP-Äquivalente benötigt, woraus sich die etwa 20% niedrigere Verwertung der Essigsäure gegenüber Milchsäure bzw. Alkohol erklärt. Trotz der hohen Streuung erscheint uns daher unter Berücksichtigung der methodischen Schwierigkeiten das Ergebnis als repräsentativ für die Abschätzung der energetischen Verwertung der Essigsäure.

Der Nettoenergiewert des Alkohols wurde mit 5,16 kcal/g, der Nettoenergiewert der Milchsäure mit 2,73 kcal/g und der Nettoenergiewert der Essigsäure mit 2,1 kcal/g bestimmt.

Damit liegen die energetischen Kennzahlen für die wichtigsten Gärungsprodukte in den Silagen vor. Nach Abschluß der z. Z. im Institut laufenden Untersuchungen zur routinemäßigen analytischen Erfassung der Gärungsprodukte ist vorgesehen, entsprechende Vorschläge für die Berechnung des energetischen Futterwertes von Silagen bei Schweinen zu unterbreiten.

Zusammenfassung

In Modellversuchen (Gesamtstoffwechselversuche) wurde die energetische Verwertung von Alkohol, Milch- und Essigsäure durch Schweine unter der Bedingung der Fettproduktion ausgewachseenr Tiere bestimmt.

Nach der Differenzauswertung wurden folgende Ergebnisse erhalten:

	Ansatz in cal/g verd. Zulage	Verwertung in % der umsetzbaren Energie
Alkohol	5164 ± 251	72,2 ± 3,4
Milchsäure	2731 ± 140	74,8 ± 4,8
Essigsäure	2100 ± 543	59,9 ± 14,7

Резюме

Опыты-модели со свиньями по определению энергетического использования спирта, уксусной и молочной кислот

В опытах-моделях (опыты по общему обмену веществ) определялось энергетическое использование спирта, молочной и уксусной кислот свиньями в условиях образования жира у взрослых животных.
С помощью дифференцированного метода были получены следующие результаты:

	прирост в кал/г. разб. прибавки	использование в % обменной энергии
спирт	5164 ± 251	72,2 ± 3,4
молочная кислота	2731 ± 140	74,8 ± 4,8
уксусная кислота	2100 ± 543	59,9 ± 14,7

Summary

Model Experiments with Pigs for Determining the Energy Utilization of Alcohol, Acetic Acid and Lactic Acid

Model experiments (total metabolism studies) were carried out to investigate the energy utilization of alcohol, acetic acid and lactic acid in full-grown fattening pigs.
Differential analysis yielded the following results:

	Energy deposition (cal/gm of the supplements)	Utilization (%) of metabolizable energy
Alcohol	5164 ± 251	72,2 ± 3,4
Lactic Acid	2731 ± 140	74,8 ± 4,8
Acetic Acid	2100 ± 543	59,9 ± 14,7

Literaturverzeichnis

[1] NEHRING, K., R. SCHIEMANN und L. HOFFMANN: Sitz.-Ber. dtsch. Akad. Landwirtsch.-Wiss. Berlin XV, H. 21, S. 19 (1966)
[2] SCHIEMANN, R., L. HOFFMANN und K. NEHRING: Arch. Tierernähr. 11, 265 (1961)
[3] WEISSBACH, F., und W. LAUBE: Z. landwirtsch. Vers.- und Unters.-Wesen 10, 65 (1964)
[4] CHARLET-LERY, G.: Proceedings of the 3rd Symp. on Energy Metabolism. EAAP-Publ. Nr. 11, 279 (1965)
[5] JENTSCH, W., L. HOFFMANN und R. SCHIEMANN: Arch. Tierernähr. 18, 344 (1968)

Eingegangen: 21. Juni 1967

Vol. 62, No. 1
Printed in U.S.A.

GASTROENTEROLOGY
Copyright © 1972 by The Williams & Wilkins Co.

PEPSIN STIMULATED BY TOPICAL HYDROCHLORIC AND ACETIC ACIDS

LEONARD R. JOHNSON, PH.D.

Department of Physiology and Biophysics, University of Oklahoma Medical Center, Oklahoma City, Oklahoma

Normally the gastric mucosa is relatively impermeable to hydrogen ions. When, however, the barrier to diffusion is broken, acid enters the mucosa, causing damage. Back diffusion of H^+ and damage in dogs produces conditions similar to those existing in patients with gastric ulcer, and it has been suggested that the primary defect in gastric ulcer disease may be an abnormally permeable gastric mucosa. Pepsin secretion during the presence of noninjurious and damaging amounts of hydrochloric and acetic acid was studied in 4 dogs with vagally denervated pouches of the oxyntic gland area. Irrigation with 200 mN HCl caused a 40% rise in pepsin output, 250 mN HCl increased pepsin output 75%, and 300 mN HCl caused a 300% increase over control levels obtained during irrigation with 100 mN HCl. Pepsin outputs were directly proportional to the flux of H^+ out of the pouch. Irrigation with 100 mN acetic acid caused a 3-fold increase in pepsin output as well as increases in Na^+ and K^+ movement into the pouch fluid and H^+ movement out. Intravenous atropine (100 μg per kg-hr) prevented the acetic acid induced increase in pepsin secretion without significantly altering ion fluxes. It is concluded that: (1) back diffusion of H^+ stimulates pepsin output, (2) a local cholinergic reflex is probably involved in this stimulation, and (3) the increased pepsin output is due to actual secretion.

The gastric mucosal barrier and the alterations in the physiology of the barrier which occur during damage provide a meaningful model of the events which may lead to the formation of a gastric ulcer. The gastric mucosa is relatively impermeable to hydrogen ions. If, however, the barrier is weakened, back diffusion of acid occurs and damage results. Davenport[1-3] has shown that damaging the canine Heidenhain pouch (vagally denervated) with acetic or salicylic acid results in excessive movement of hydrogen ion out of the pouch and increased flux of sodium and potassium into the lumen. In addition, fluid accumulates in the pouch along with plasma protein.[4] If damage is severe, bleeding occurs. During damage, histamine is released into the mucosa[5] and the activity of the enzyme which forms histamine increases.[6] The higher levels of intramucosal histamine probably add to the damage by causing edema, fluid production, and increased mucosal permeability and acid secretion.

It has recently been shown that pepsin output increases when pouches are dam-

Received July 8, 1971. Accepted August 5, 1971.
These results were presented in part to the American Gastroenterological Association, Miami, Florida, May 1971.

Address requests for reprints to: Leonard R. Johnson, Ph.D., Department of Physiology and Biophysics, 800 N.E. 13th Street, Oklahoma City, Oklahoma 73104.

This study was supported by a grant from the G. A. Manahan Trust Fund and by National Institutes of Health Grant AM-14392.
The expert technical assistance of Louise H. Wyss is gratefully acknowledged.

aged by high concentrations of hydrochloric acid in the presence of a subthreshold (for acid secretion) infusion of pentagastrin.[7] This study demonstrates that this increased pepsin output takes place in the absence of gastrin, is related to the back diffusion of hydrogen ion, and is secretory in nature.

Methods

Four dogs were surgically prepared with a gastric fistula drained by a Thomas[8] cannula and a vagally denervated (Heidenhain) pouch with a Gregory[9] cannula. These particular experiments were begun about 2 years after surgery. The animals were fasted for at least 18 hr but were allowed water before an experiment. At the start of an experiment the dogs were placed on tables where they stood quietly supported, in part, by slings. The gastric fistula was opened and the stomach was rinsed with saline. The fistula was left open for the duration of the experiment to prevent gastric juice from entering the duodenum. The metal cannula of the Heidenhain pouch was connected by rubber tubing to a reservoir with 50 ml of a control solution containing 100 mN HCl and 50 mN NaCl. The solution was allowed to enter and leave the pouch influenced only by gravity and pouch contractions. The level of fluid in the reservoir was about 20 cm above the pouch. At the end of each 15-min period the reservoir and the pouch were drained and the solution was replaced with 50 ml of fresh acid. Volumes of recovered fluid ranged from 48 to 51 ml, and, after the first two 15-min periods, which were not included in the experiment, were usually 50 ml.

Collections of the control solution continued for four 15-min periods. The pouches were then irrigated with 200 mN HCl, 250 mN HCl, 300 mN HCl, or 100 mN acetic acid plus 50 mN NaCl for four periods. In each case the 100 mN HCl control solution was made isosmotic with the test solution by the addition of NaCl. A final hour of collections with the control solution ended the experiment. At the beginning of an experiment in the acetic acid series an intravenous infusion of saline was started in a leg vein and maintained for the duration of the experiment at a rate of 60 ml per hr by a Harvard peristaltic pump. On some days atropine was added to the infusion to give a dose of 100 μg per kg-hr. Other experiments consisted entirely of irrigating the pouch with the control solution.

After each 15-min collection the volume of the sample was measured and the acid concentration was determined by titration to pH 7.0 with 0.2 N NaOH on an Autoburette and pH meter (Radiometer, Copenhagen). The Na^+ and K^+ concentrations of each sample were determined by flame photometry (IL model 143, Instrumentation Laboratory, Inc., Lexington, Mass.). A volume of each sample was immediately diluted so that it contained 50 mN HCl. The pepsin concentration of this sample was determined using a modification[10] of the Anson[11] hemoglobin method and expressed as milligrams of pepsin per milliliter by reading the trichloroacetic acid supernatant fluid at 280 mμ and comparing it with solutions incubated with different pepsin standards (Hog pepsin, three times crystallized, Pentex Biochemicals, Kankakee, Ill.). Pepsin outputs were calculated, taking into consideration the dilution of the assay, and were expressed as milligrams of pepsin per 15 min. The data from the experiments with hydrochloric acid were expressed as the percentage of the mean of the two control periods during which the pouches were irrigated with 100 mN HCl and 50 mN NaCl. This was done to eliminate some of the variability between individual animals which had consistent but different basal levels of pepsin secretion. The mean pepsin value of the periods taken as 100% was 2.8 mg per 15 min.

Total amounts of Na^+, K^+, and H^+ in the recovered fluid were calculated for each period during the acetic acid experiments. The net flux of each ion was determined by subtracting the amount put in the pouch from the amount recovered and expressed as microequivalents per 15 min. Negative numbers indicate a net loss of an ion from the pouch, i.e., mucosal to serosal movement.

Experiments were done on alternate days and no more than three times a week. Within each group of experiments the order was randomly assigned. Results of the HCl study represent the means of three observations in each of four dogs. Following the completion of this series 1 animal developed a leaky pouch and was dropped from the study. Therefore, the acetic acid experiments were run on 3 animals and the data are expressed as the means and standard errors of the means of nine observations.

Results

Irrigation of the pouches with 200 and 250 mN HCl significantly increased pepsin output over that seen in response to 100 mN HCl ($P < 0.05$, periods 4 and 5, fig. 1). Irrigation with 300 mN HCl produced a

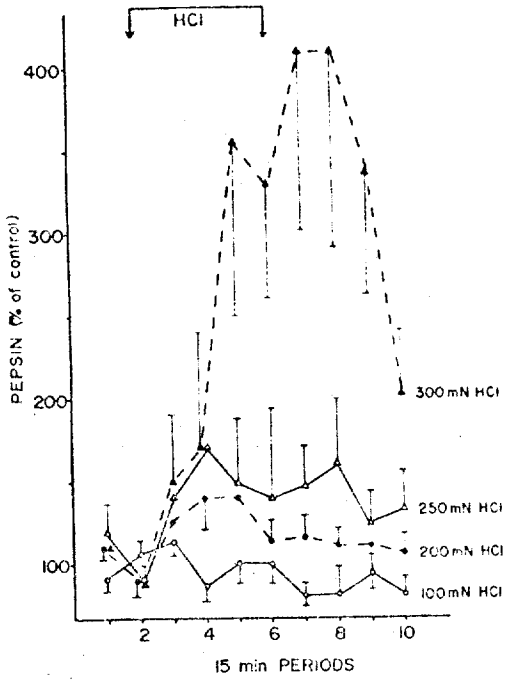


FIG. 1. Pepsin secretion as percentage of control from vagally denervated canine pouches. During periods 3 to 6 the pouches were irrigated with the concentrations of HCl indicated in the *margin*. During the remaining time, the irrigation solution contained 100 mN HCl plus NaCl to make the solution isotonic with the acid during periods 3 to 6. Means and standard errors of the means are shown for three observations in each of 4 dogs.

dramatic 3- to 4-fold increase in pepsin secretion. It can be seen in figure 1 that pepsin secretion was approximately the same during the control periods even though the osmolarity of the solutions differed. These relatively high concentrations of HCl were used to increase the diffusion gradient for H^+, thereby causing a flux of H^+ through the normal mucosa approximately equivalent to what would be expected if the barrier had been broken by an extraneous agent such as alcohol, acetic acid, or aspirin. These agents were avoided at the outset of the experiments in order to be able to attribute any stimulation in pepsin secretion to H^+ itself without having to worry about unphysiological anions or substances like alcohol which could stimulate on their own.

Pepsin secretion was directly proportional to the concentration of acid placed in the pouches for 100, 200, and 250 mN HCl (fig. 2). At 300 mN HCl this relationship no longer existed for pepsin output increased out of proportion to the increase in acidity. When, however, the data were replotted, pepsin secretion was found to be directly related to the net flux of hydrogen ion out of the pouch (*solid line*, fig. 2; $r = 0.997$). Thus, in addition to a large increase in pepsin secretion in going from 250 to 300 mN HCl, one also finds a concurrent increase in H^+ flux.

Irrigation of the pouches with 100 mN acetic acid (fig. 3) caused a 3-fold increase

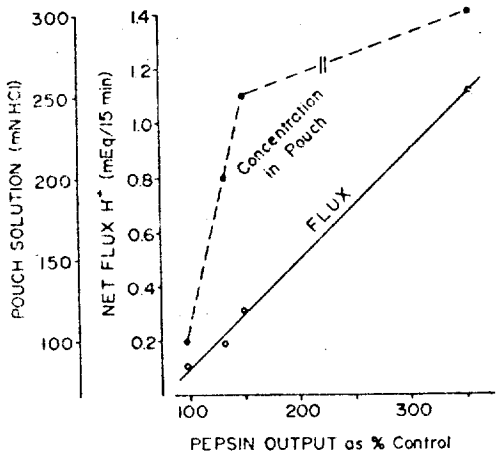


FIG. 2. Peak pepsin outputs from figure 1 plotted against the concentration of acid in the irrigating solutions and against the net flux of H^+ out of the pouch during the corresponding period.

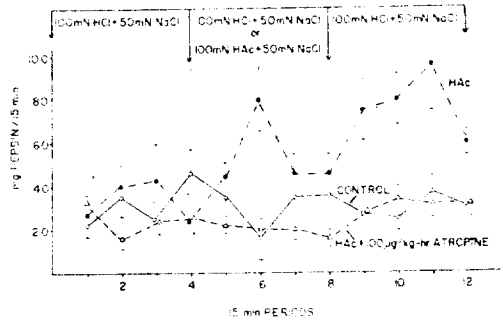


FIG. 3. Pepsin outputs in response to control period and irrigation with 100 mN acetic acid solutions. In one set of experiments atropine was infused intravenously. Means and standard errors of the means of three observations in each of 3 animals are shown.

in pepsin output over control periods before and during acetic acid irrigation (i.e., periods 4 and 6, $P < 0.01$). Following acetic acid irrigation, 100 mN HCl caused a further rise in pepsin secretion ($P < 0.01$, periods 9, 10, 11, 12) indicating that the barrier to ion diffusion had been disrupted by the acetic acid. Figure 3 also demonstrates that 100 μg per kg-hr atropine infused intravenously for the duration of the experiment completely blocked the increase in pepsin secretion in response to acetic acid.

The ability of acetic acid to damage the mucosal barrier was relatively unaffected by atropine. This is shown by the similarity in the volume changes and fluxes of Na^+, K^+, and H^+ during intravenous saline

TABLE 1. *Net changes across Heidenhain pouches of 3 dogs during intravenous saline infusion*[a]

Solution	Period	Volume	Na	K	H^+
		ml		*μEq*	
Control: 100 mN HCl and 50 mN NaCl	1	0	159	5	−654
	2	0.5	−66	4	−381
	3	0.3	−79	3	−302
	4	0.5	25	2	−287
100 mN acetic acid and 50 mN NaCl	5	1.7	287	16	−653
	6	1.7	460	30	−916
	7	1.7	472	29	−950
	8	0.8	395	26	−914
100 mN HCl and 50 mN NaCl	9	1.3	404	21	−892
	10	1.0	334	18	−715
	11	1.3	326	15	−593
	12	2.2	364	14	−521

[a] Net volume and ion fluxes per 15-min period during the acetic acid experiments shown in figure 3. Saline was infused intravenously throughout. Numbers are means of three observations in each of 3 animals. Negative numbers indicate mucosal to serosal flux.

TABLE 2. *Net changes across Heidenhain pouches of 3 dogs during intravenous infusion of atropine (100 μg per kg-hr)*[a]

Solution	Period	Volume	Na	K	H^+
		ml		*μEq*	
Control: 100 mN HCl and 50 mN NaCl	1	0.3	200	10	−549
	2	0.1	50	7	−266
	3	0.1	44	6	−307
	4	0.3	85	5	−325
100 mN acetic acid and 50 mN NaCl	5	0.7	248	20	−600
	6	1.3	425	26	−786
	7	0.7	367	24	−768
	8	0.8	334	21	−703
100 mN HCl and 50 mN NaCl	9	0.4	279	20	−894
	10	0.6	281	20	−608
	11	0.7	241	16	−624
	12	1.0	233	15	−519

[a] Same as table 1 except that data were taken from the experiments in figure 3 in which atropine was infused throughout.

(table 1) and atropine (table 2) infusion. In other words, the pepsin output was the only entity altered by atropine.

Discussion

A previous study demonstrated that high concentrations of HCl could increase pepsin output in the presence of a background intravenous infusion of gastrin.[7] This work shows that pepsin output increases in response to the back diffusion of H^+ in the absence of gastrin stimulation. If the primary defect in some cases of gastric ulcer is, as Davenport[12] suggests, an abnormally permeable gastric mucosa, the concurrent increase in pepsin output could play a significant causative role in the production of gastric ulcers.

Large increases in pepsin output did not occur in response to HCl until sufficient acid was used to break the mucosal barrier to back diffusion. As is readily apparent in figure 2 the flux of H^+ did not increase out of proportion to the concentration put into the pouch until 300 mN HCl was used. Therefore, the threshold concentration of HCl which damages the barrier is somewhere between 250 and 300 mN. This is the same approximation arrived at by Davenport.[12]

The increased pepsin output in response to nondamaging concentrations of HCl is extremely interesting from the standpoint of the physiological control of pepsin secretion. These data indicate that the normal gastric mucosa contains a mechanism sensitive to H^+ which stimulates pepsin secretion, and we are currently involved in experiments to determine whether pepsin secretion is stimulated by the presence of physiological amounts of acid within the pouches.

The large increase in pepsin output in response to 100 mN acetic acid (fig. 3) indicates that the effect is not peculiar to damage by high (300 mN) concentrations of hydrogen ion. As Davenport[3, 4] has previously demonstrated, this concentration of acetic acid breaks the barrier to ion diffusion, resulting in the loss of H^+ from the pouch and a net gain of Na^+ and K^+ similar to what was observed in the current experiments (table 1). Although the barrier was broken by a much lower concentration of acetic acid than HCl, the relationship between pepsin output and H^+ back diffusion still holds. During acetic acid irrigation maximal pepsin secretion was roughly 300% greater than the control. This corresponded to a back diffusion of 916 μEq of H^+. In the flux curve drawn for HCl and pepsin output (fig. 2) a 300% increase in pepsin corresponds to the back diffusion of 900 μEq of H^+.

The observation that atropine blocks pepsin output during damage by acetic acid without affecting ion fluxes indicates that the increased output of pepsin is due to actual secretion. Since damage still occurs with atropine, the absence of the pepsin effect dissociates the source of pepsin from damage per se, that is, sloughed or broken cells. This observation also indicates the involvement of a local intramural cholinergic reflex in the stimulation of pepsin secretion.

From these experiments we conclude that (1) the back diffusion of H^+ increases the output of pepsin into the canine Heidenhain pouch, (2) the amount of pepsin secreted is directly related to the amount of H^+ lost from the pouch, (3) the increased pepsin output represents actual secretion, and (4) a cholinergic reflex is involved in the stimulation of this secretion. In addition, it is tempting to speculate that normal amounts of HCl in the stomach may actually enhance the physiological secretion of pepsinogen as well as activate it.

REFERENCES

1. Davenport HW: Gastric mucosal injury by fatty and acetylsalicylic acids. Gastroenterology 46: 245-253, 1964
2. Davenport HW: Damage to the gastric mucosa: effects of salicylates and stimulation. Gastroenterology 49:189-196, 1965
3. Davenport HW: Potassium fluxes across the resting and stimulated gastric mucosa: injury by salicylic and acetic acids. Gastroenterology 49: 238-245, 1965
4. Davenport HW: Fluid produced by the gastric mucosa during damage by acetic and salicylic acids. Gastroenterology 50:487-499, 1966

5. Johnson LR, Overholt BF: Release of histamine into gastric venous blood following injury by acetic or salicylic acid. Gastroenterology 52: 505-509, 1967
6. Johnson LR: The source of histamine released during damage to the gastric mucosa by acetic acid. Gastroenterology 54:8-15, 1968
7. Johnson LR: Pepsin output from the damaged canine Heidenhain pouch. Am J Dig Dis 16:403-407, 1971
8. Thomas JE: An improved cannula for gastric and intestinal fistulas. Proc Soc Exp Biol Med 46: 260-261, 1941
9. Gregory RA: Gastric secretory responses after portal venous ligation. J Physiol (Lond) 144: 123-127, 1958
10. Northrup JH, Kunitz, M, Herriot RM: Crystalline Enzymes. Second edition. New York, Columbia University Press, 1948, p 303-307
11. Anson ML: The estimation of pepsin, trypsin, papain and cathe pepsin with hemoglobin. J Gen Physiol 22:79-89, 1938
12. Davenport HW: Back diffusion of acid through the gastric mucosa and its physiological consequences. Progress in Gastroenterology, vol 2. Edited by GBJ Glass. New York, Grune and Stratton, 1970, p 42-56

Gastroenterology 54: 8-15; (JAN. 1968)

SOURCE OF THE HISTAMINE RELEASED DURING DAMAGE TO THE GASTRIC MUCOSA BY ACETIC ACID

LEONARD R. JOHNSON, PH.D.

Department of Physiology, The University of Michigan, Ann Arbor, Michigan

Many investigators have suggested that during stress histamine is released from the gastric mucosa and is responsible in part for ensuing gastric erosions.[1-3] We have obtained direct evidence that histamine is released during damage and is present in the gastric mucosal interstitial fluid where it may exert its characteristic effects.[4, 5] When the rat gastric mucosa is irrigated with 100 mM acetic acid or 20 mM salicylic acid in 100 mM HCl, histamine appears in the gastric contents, and the histamine content of the oxyntic glandular area simultaneously decreases.[4] These changes do not occur when the stomachs are filled with 154 mM NaCl or 100 mM HCl. Histamine appears in the gastric venous blood of dogs whose stomachs are damaged with acetic or salicylic acid.[5] Histamine release begins within 5 min of initiation of damage and is complete after 30 min. No histamine is released into the venous effluent when the stomachs are filled with 154 mM NaCl or 100 mM HCl. Large amounts of histamine also appear in the contents of damaged dog stomachs, but not in the contents of stomachs from control animals.[5] Again, all of the histamine is released during the first 30 min of injury. We have concluded that the decreased mucosal histamine and the appearance of histamine in gastric contents and gastric venous blood during damage are proof that histamine is released into the mucosal interstitial fluid.

Histamine released within the gastric mucosa could come from several sources. First, there is a pool of mast cell histamine, which is released by compound 48/80.[6] Release occurs quickly with degranulation and replenishment is slow. Second, there is a pool of non-mast cell histamine, probably associated with the parietal cells, accounting for approximately 70% of the total amount present.[7] This is not released by 48/80. It quickly exchanges with circulating histamine and is released slowly.[7] In addition, the histidine decarboxylase of the gastric mucosa is adaptive and, under a variety of circumstances, capable of a several-fold increase in activity.[8] Presumably, an increase in the histamine-forming capacity (HFC) could supply histamine for physiological functions.

This report concerns an investigation of the source of the histamine released from the stomach during damage. In order to differentiate between the various supplies of histamine available, the histamine-forming capacities of damaged rat stomachs have been compared with those of controls, and histamine release during mucosal injury has been measured after pretreating rats with compound 48/80.

Methods

Experimental procedure. Male rats weighing between 200 and 400 g were fasted overnight without being restrained. Each rat was anesthetized for the duration of the experiment with sodium pentobarbital. The stomach was exposed by midline incision and ligated at the pylorus. The stomach was rinsed twice with

Received June 23, 1967. Accepted August 22, 1967.

Address requests for reprints to: Leonard R. Johnson, Ph.D., Building 114, Room 231, Veterans Administration Center, Los Angeles, California 90073.

This investigation was supported by Research Grant AM-08716 from the United States Public Health Service.

Dr. Johnson was supported by a Predoctoral Fellowship, United States Public Health Service.

The author is grateful to Dr. H. W. Davenport for continued advice and support.

154 mM NaCl injected and removed through the forestomach. Then either 154 mM NaCl or 100 mM acetic acid plus 54 mM NaCl was injected at volume equal to 1.5% of body wt. The fluid was left in the stomachs for various times after which the animal was killed by a blow on the head and the stomach was immediately removed.

Extraction and determination of histamine. The stomach content was measured to the nearest 0.1 ml and at once combined with 5 ml of concentrated HCl to begin the histamine extraction procedure described by Code and McIntire.[9] Histamine content of the final extracts was determined by assay using an isolated terminal segment of guinea pig ileum suspended in atropinized Tyrode's solution.[10] Potassium concentration of the final extracts were measured by internal standard flame photometry and found to be well below the level which would affect the isolated ileum.[11] Contractions attributed to histamine were abolished by 1.0 mg per liter of pyrilamine maleate. All results are expressed as micrograms of histamine base. The minimal amount of detectable histamine was 0.001 μg per stomach. The percentages recovered of histamine added to the irrigation fluids were 75.0 for acetic acid and 74.5 for NaCl. No corrections were made for incomplete recovery.

Measurement of the histamine-forming capacity. After removing the fluid, the glandular portion of a stomach was isolated and immediately frozen in liquid nitrogen. The frozen glandular stomach was powdered in a mortar and then combined with the powdered stomachs from several other identically treated rats. The frozen powder was emptied into a conical, glass homogenizer containing 0.1 M phosphate buffer (*p*H 7.0) and homogenized. The brei was transferred to a cylindrical homogenizer, and the process repeated. Following transfer to a centrifuge tube, the homogenate was spun at 20,000 × *g* for 20 min at 4 C. The resulting cell-free solution was used as the source of the enzyme, histidine decarboxylase. The protein concentration of the enzyme preparation was determined by biuret analysis according to the procedure outlined by Layne.[12]

Two milliliters of enzyme solution, 30.0 μg of pyridoxal phosphate, 200.0 μg of *l*-histidine, 0.3 μc of *dl*-carboxyl ^{14}C-histidine, and phosphate buffer (*p*H 7.0) to a final volume of 2.65 ml were added to a Warburg type flask. A 6 by 25 mm test tube containing 0.3 ml of 1 M hyamine hydroxide was placed in the center well. A small strip of filter paper was inserted into the tube to increase the surface area of the hyamine. Flasks were sealed with sleeve type, needle puncture, rubber stoppers and their contents were incubated for 3 hr with constant agitation at 37 C. The reaction was stopped with 0.25 ml of 1 N HCl, injected through the rubber stopper. Flasks were agitated 1 additional hr to allow complete absorption of $^{14}CO_2$.

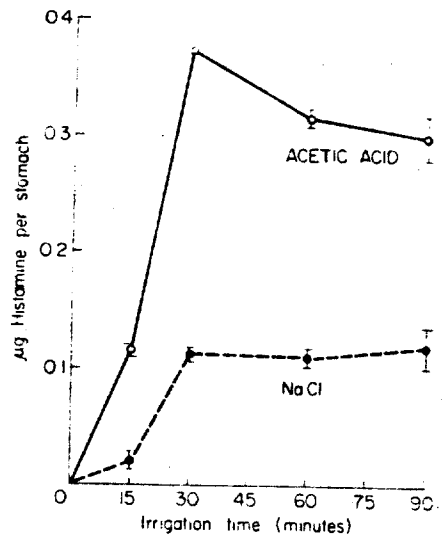


FIG. 1. Histamine found in rat gastric contents at different times after filling with NaCl or acetic acid (*μg Histamine base-vertical lines* are standard errors of the means). *Lines* are significantly different. $P < 0.01$.

This method depends on the release of $^{14}CO_2$ from carboxyl-labeled histidine giving one molecule of CO_2 for each molecule of histamine formed and was originally described by Kobayashi.[13] Counts per minute of $^{14}CO_2$ were determined in a Nuclear-Chicago 720 liquid scintillation system. Disintegrations per minute were calculated from efficiency curves, the percentage conversion of histidine to histamine was determined, and the histamine-forming capacities were expressed as nanomoles of histamine formed per 3 hr per 100 mg of protein.

Results

Histamine released into the gastric contents. Much more histamine was present in the acetic acid irrigation fluids than in the NaCl fluids at all times following filling the stomachs (fig. 1). Release occurred

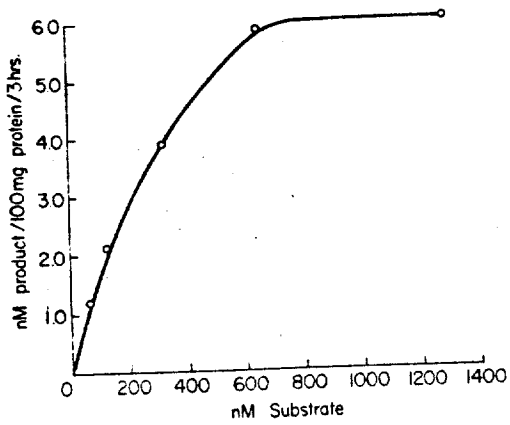


FIG. 2. Determination of optimal substrate concentration for measurement of the histamine-forming capacity. Graph represents data from glandular mucosa of 5 rats.

quickly, was maximal during the second 15-min period, and was complete after 30 min of irrigation. Thus, stomachs filled for 90 min released no more histamine than those filled for 30 min. In fact, the contents of stomachs injured with acetic acid contained less histamine after 90 min. This disappearance, which was not seen with NaCl, was probably due to a back diffusion of the amine through the damaged, permeable mucosa.

If, after 30 min of irrigation with acetic acid, the fluid was carefully removed and the stomach was refilled with fresh acetic acid, no, or very little, additional histamine appeared. This means that release was complete after 30 min of exposure, and failure to find additional histamine after 60 or 90 min was not due to the disappearance of a concentration gradient of histamine between the interstitial fluid and the contents of the lumen.

Histamine formation. The formation of histamine was determined in the presence of varying amounts of histidine. The optimal substrate concentration for the system was about 1.2 μM of histidine per incubation flask (fig. 2). The cofactor, pyridoxal phosphate, 30 μg per flask, was required for maximal enzyme activity of preparations from both damaged and control stomach tissue. Histamine formation was optimal in the presence of phosphate buffer, *p*H 7.0. These conditions were used throughout the series of experiments. The HFC of stomachs irrigated for 60 min with NaCl was 13.9 nmoles per 3 hr per 100 mg of protein ±1.5 (SE) when incubated in the presence of pyridoxal phosphate and 8.4 ± 0.6 when incubated without the cofactor ($P < 0.05$). The corresponding values for stomachs damaged with acetic acid were 32.7 ± 0.1 and 12.6 ± 1.6 ($P < 0.01$).

Histamine formation was directly proportional to the incubation time and the amount of enzyme present (fig. 3). The data, therefore, may be legitimately expressed as functions of enzyme concentration and incubation time.

Pylorus-ligated rat stomachs were filled for various lengths of time with either NaCl or acetic acid, the glandular areas were excised, and their ability to form histamine in vitro was determined. At all times after the beginning of irrigation, the HFC of the damaged stomachs was almost double that of the stomachs filled with NaCl (fig. 4). Within 15 min, the HFC of stomachs treated with acetic acid increased 3-fold; this increase was 73.5% greater than that seen with NaCl. At the end of 2 hr of irrigation with acetic acid, enzyme activity was increased 12-fold; the increase was only 6-fold in the stomachs filled with NaCl. Figure 4 represents 12 experiments using 74 rats; in each experiment the HFC of each injured rat was greater than that of every control animal.

Two features of figure 4 deserve explanation. First, the two lines are amazingly parallel, probably because paired rats were used in each experiment; that is, the animals used for one experiment were littermates of the same size, exposed to the same conditions of temperature, light, etc. Therefore, when the HFC of the injured rats was especially high or low, the values found for the control animals in the same experiment were correspondingly high or low. Second, although the increase seen with NaCl was only half that seen with acetic acid, it was still a substantial increase in enzyme activity. When gastric

secretion is stimulated, the HFC increases[8]; this probably explains the increased HFC seen in the stomachs filled with NaCl. Pylorus ligation stimulates gastric secretion and large increments in volume and H^+ concentration were observed in control experiments. In the one experiment in which this was tested, the HFC of empty pyloric-ligated stomachs was 95% of the HFC of pyloric-ligated, NaCl-filled stomachs.

The initial increase in the HFC occurs at the same time as histamine release. But, whereas release is complete within 30 min, enzyme activity remains high for the duration of irrigation. In fact, the highest HFC values were obtained when no histamine was being released (fig. 5). This suggests that the increase in the ability to form histamine does not account for the histamine which is quickly released into the lumen. It is more probable that release triggers an increase in histamine formation.

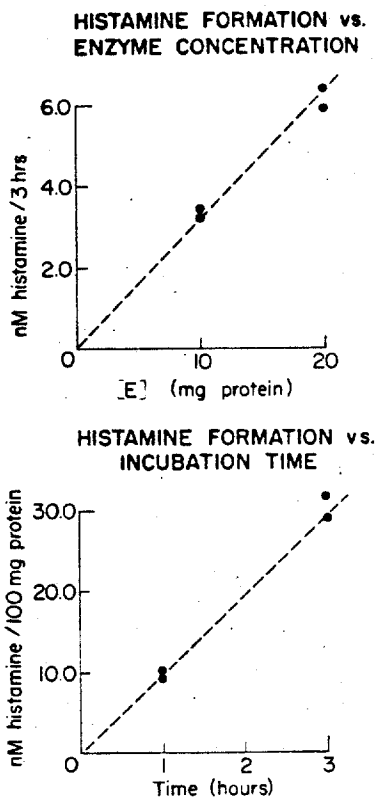


FIG. 3. Graphs showing that histamine formation is directly proportional to enzyme concentration (*top*) and incubation time (*bottom*). In both cases, *0* is a true point on the *line*.

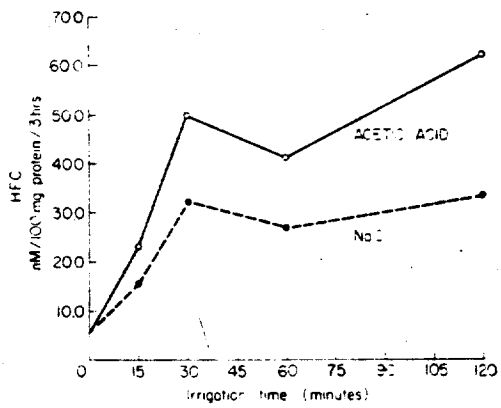


FIG. 4. Comparison of the effects of irrigation with acetic acid and NaCl on the ability of rat gastric mucosa to form histamine. Stomachs from the same animals represented in figure 1. There was no overlap in these experiments.

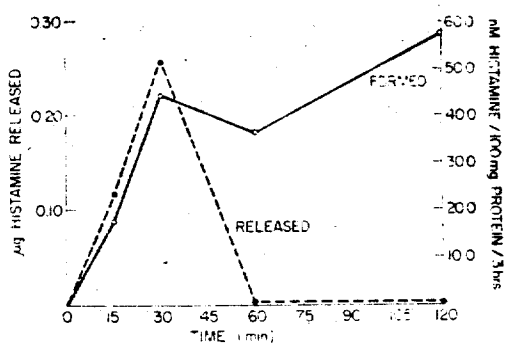


FIG. 5. Superimposed curves of the time course of histamine release and the increase in the ability to form histamine. Rat stomachs were damaged with acetic acid irrigation.

Experiments with compound 48/80. The experiments described above indicate that the increased HFC does not supply the histamine detected during damage. The fact that release occurs quickly and is of short duration is indicative of mast cell degranulation.[14] To test this hypothesis rats were injured with acetic acid after depleting mast cell histamine with compound

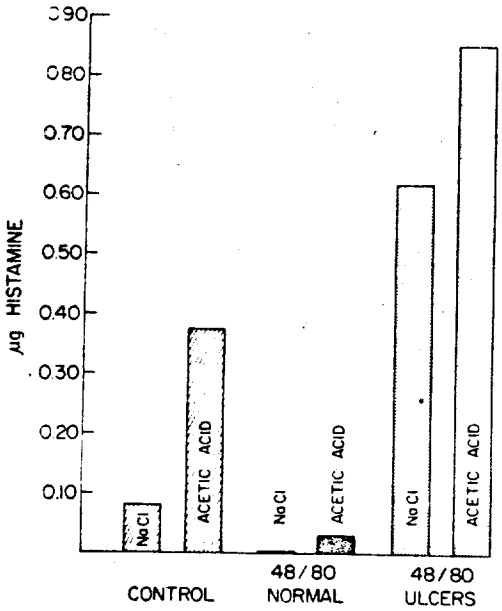


FIG. 6. Comparison of the effects of 30-min irrigation with acetic acid on histamine release in normal fasted rats and in rats pretreated with compound 48/80. The 48/80 rats are separated into two populations—those that did not develop ulcers and those that did. Each *bar* represents a mean taken from data on at least 6 rats. Each of the three groups is significantly different from the other two with $P < 0.01$. Within the control group, NaCl and acetic acid means are significantly different, $P < 0.01$. The two *bars* comprising the 48/80 rats with ulcers do not differ significantly.

48/80. The histamine content of NaCl and acetic acid irrigation solutions was determined after 30 min of contact with the gastric mucosa.

Injection with 48/80 almost completely abolished the difference between the amounts of histamine found in NaCl and acetic acid irrigation fluids (fig. 6). Some of the rats treated with 48/80 developed massive gastric ulcers, and these released large amounts of histamine into the gastric contents. Without exception, 48/80-treated rats with normal appearing gastric mucosae failed to produce significant amounts of histamine. Two conclusions can be drawn from this experiment. First, the histamine, which is quickly released by acetic acid during injury and which accounts for the 4-fold difference in the histamine content of the NaCl and acetic acid irrigation fluids, is probably of mast cell origin. Second, after injury has become substantial and long lasting, histamine is again released from the gastric mucosa, but not from the mast cells.

Discussion

Three of the results reported here are of particular significance in establishing the mechanism of histamine release from the damaged stomach. First, histamine release from damaged stomachs of rats occurs early and is of brief duration—the same as in dogs.[5] Second, mast cells are probably the source of the released histamine. Third, during damage histamine formation increases. These three findings and their significance in the injury process are depicted in figure 7. Acid diffusing through the permeable gastric mucosa damages the tissue, and histamine is released into the interstitial fluid. Several lines of results—both direct and indirect—point to the mucosal mast cells as being the source of the histamine released during damage. The concentration of histamine in the rat's gastric mucosa decreases during damage.[4] The HFC is high during periods when release is not occurring (fig. 5). Release appears to be identical in the rat and dog with regard to onset and duration; the enzyme, histidine decarboxylase, has been found only in the rat gastric mucosa. These findings imply a release of preformed, rather than newly synthesized, histamine. In both dogs and rats release begins shortly after the initiation of damage and is complete after 30 min. These results are characteristic of mast cell release. Pretreatment with compound 48/80 reduces the binding of ^{3}H-histamine by rat gastric mucosa approximately 30%.[7] This corresponds to the reduction with salicylic and acetic acids.[4]

The case for the mast cells supplying the histamine released during damage is well supported. There is, however, another explanation which fits the data. If, in the intact animal, the supply of histidine is limited and the HFC is providing the hista-

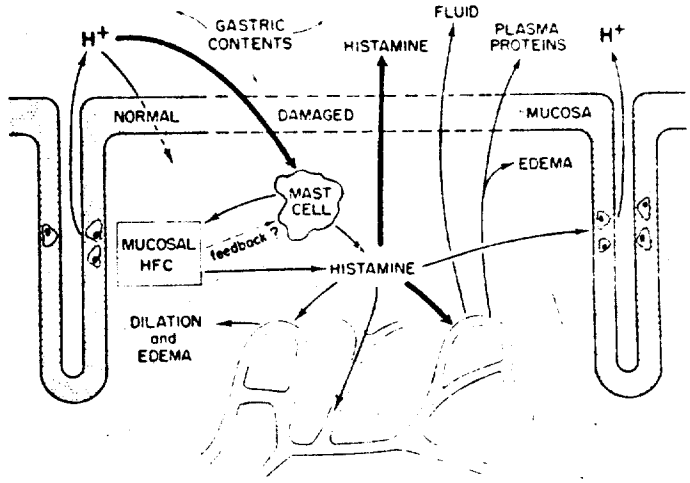


FIG. 7. Current concept of histamine release and formation during damage and how it can account for the changes observed.

mine during damage, histamine will be released only as long as substrate is available. In the incubation flask, however, there is unlimited substrate, and the induced enzyme is able to begin where it left off in vivo. The experiments with 48/80 make this alternative possibility highly unlikely, but it is a possible explanation.

What is the function of the increased activity of the histamine-forming enzyme? The obvious answer is to replace the histamine lost during release. Kahlson, however, has accumulated considerable evidence that histamine may be a type of growth hormone for rapidly growing tissues.[15] The highest HFC's are observed in fetal rat livers, wound tissue, and certain malignant growths. All of these are characterized by extremely fast growth. In the stomach, this scheme is complicated by the fact that histamine stimulates acid secretion and can cause ulcer formation.

Some of the rats treated with compound 48/80 developed large ulcers. Most of these animals were large (400 g), while, as a rule, the stomachs of the smaller rats (200 g) appeared normal. The correlation of the size of the rats with ulcerogenesis may have been due to the greater amounts of histamine reaching the stomachs of the larger animals from degranulated mast cells of the skin and other tissues. The ulcerated stomachs released large amounts of histamine into both NaCl and acetic acid irrigation fluids. This histamine was diffusing through the damaged mucosa and was not related to the composition of the fluid in the stomach. By this time, the mast cells of the entire body were almost 100% degranulated. This means that histamine released after damage is well established comes from a source other than the mast cells. This could be a release of non-mast cell histamine stores, but this seems unlikely for the amount being released is great, and release no doubt continues over a long period of time. It is more probable that histamine appearing during the late phase of damage represents the contribution of the elevated HFC. These results imply that histamine is present to exert its effects after injury is well established as well as during the early phase.

The pool of released histamine (fig. 7) is responsible for many of the components of damage observed in Davenport's studies.[16] Some histamine increases mucosal permeability further allowing H^+ ions to diffuse into the tissue. Histamine, itself, is

able to move through the disrupted barrier into the gastric lumen. Some histamine causes vasodilation of the mucosal capillaries increasing capillary permeability. This results in edema and the swollen appearance of the damaged rat stomachs. The increased capillary and mucosal permeability is dramatically shown by the presence of an abnormal amount of plasma protein in the gastric contents of dogs.[16] Fluid production increases greatly when the dog's stomach is damaged.[16] The increase is mainly an ultrafiltrate of plasma, but there is some gastric secretion. The component supplied by secretion is difficult to measure since H^+ ions are rapidly diffusing out of the lumen. However, when the large amount of histamine released into the mucosa is considered, one is justified in stating that some of it reaches the parietal cells and stimulates secretion. The production of H^+ ions exacerbates the damage. All of the observations in figure 7 attributed to the action of histamine occur during the time histamine is being released.

Fluid production, edema, increased mucosal permeability, and other signs of damage persist long after histamine can be detected in the gastric venous blood or contents. Little is known about the rate of repair of the damaged mucosa, but capillary permeability must remain elevated from the initial release of histamine and for some time after release is complete. It is probable, therefore, that the continued manifestations of damage are due, in part, to the large burst of histamine released initially.

Summary

The stomachs of pylorus-ligated rats were filled with 154 mM NaCl or 100 mM acetic acid plus 54 mM NaCl. After various intervals, the stomach contents were removed and the amount of histamine present was determined by bioassay on the guinea pig ileum. Homogenates were made from some rat stomachs. These were centrifuged, and the supernatant fluids were used as preparations of the enzyme, histidine decarboxylase. The activity of the enzyme was determined by counting the $^{14}CO_2$ evolved when ^{14}C-carboxyl histidine was incubated with the enzyme. This measurement was converted to the histamine-forming capacity (HFC).

Acetic acid damage released significant amounts of histamine into the gastric contents. Release was complete after 30 min of irrigation. After 15 min of irrigation the HFC of the rats treated with acetic acid was increased 3-fold and was twice that of the NaCl-treated controls. After 2 hr the HFC of damaged stomachs had increased 12-fold as compared with a 6-fold increase in the control animals. Rats were treated with the mast cell histamine depletor, compound 48/80, and their stomachs were filled with either NaCl or acetic acid. At the end of 30 min there was no histamine present in NaCl irrigation fluids and less than one-tenth the amount normally found in the contents of acetic acid damaged stomachs.

Since the releasable histamine pool is exhausted within 30 min, 48/80 prevents histamine release, and the HFC remains high after release is complete, it is concluded that the mucosal mast cells are the source of the histamine. It is hypothesized that the histamine release triggers an adaptive increase in the HFC. The increased enzyme activity furnishes more histamine to the damaged mucosa. Since activity is high throughout injury, the elevated HFC may serve some function during later stages of damage.

REFERENCES

1. Clark, B. B., and W. L. Adams. 1947. The effect of acetylsalicylic acid on gastric secretion. Gastroenterology *9:* 461–465.
2. Räsänen, T. 1961. Effect of prednisolone and deoxycorticosterone, administered separately and with growth hormone, on the mucosal mast cells and tissue eosinophils of rat stomach wall. Gastroenterology *40:* 232–234.
3. Guth, P. H., and P. Hall. 1966. Microcirculatory and mast cell changes in restraint-induced gastric ulcer. Gastroenterology *50:* 562–570.
4. Johnson, L. R. 1966. Histamine liberation by gastric mucosa of pylorus-ligated rats damaged by acetic or salicylic acids. Proc. Soc. Exp. Biol. Med. *121:* 384–386.
5. Johnson, L. R., and B. F. Overholt. 1967. Re-

lease of histamine into gastric venous blood following injury by acetic or salicylic acid. Gastroenterology *53:* 505–509.

6. Mota, I., W. T. Beraldo, A. G. Ferri, and L. C. V. Junqueira. 1956. Action of 48/80 on the mast cell population and histamine content of the wall of the gastrointestinal tract of rats, p. 47–50. *In* G. E. W. Wolstenholme and C. M. O'Connor, [eds.], Ciba Foundation Symposium on histamine. Little, Brown & Company, Boston.
7. Johnson, H. L., M. A. Beaven, F. Erjavec, and B. B. Brodie. 1966. Selective labeling and release of non-mast cell histamine. Life Sci. *5:* 115–123.
8. Kahlson, G., G. E. Rosengren, D. Svahn, and R. Thunberg. 1964. Mobilization and formation of histamine in gastric mucosa. J. Physiol. (London) *174:* 400–416.
9. Code, C. F., and F. C. McIntire. 1956. Quantitative determination of histamine, p. 49–95. *In* D. Glick [ed.], Methods of biochemical analysis, Vol. 3. Interscience Publishers, Inc., New York.
10. Barsoum, G. S., and J. H. Gaddum. 1935. Pharmacological estimation of adenosine and histamine in the blood. J. Physiol. (London) *85:* 1–14.
11. Code, C. F., G. A. Hallenbeck, and R. A. Gregory. 1947. Histamine content of canine gastric juice. Amer. J. Physiol. *151:* 593–605.
12. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins, p. 450. *In* S. P. Colowick and N. O. Kaplan [eds.], Methods in enzymology, Vol. 3. Academic Press, Inc., New York.
13. Kobayashi, Y. 1963. Isotopic method of histamine forming capacity determination. Anal. Biochem. *5:* 284–285.
14. Paton, W. D. M. 1951. Compound 48/80: a potent histamine liberator. Brit. J. Pharmacol. *6:* 499–507.
15. Kahlson, G. 1962. New approaches to the physiology of histamine. Perspect. Biol. Med. *Winter:* 179–197.
16. Davenport, H. W. 1966. Fluid produced by the gastric mucosa during damage by acetic and salicylic acids. Gastroenterology *50:* 487–499.

Journal of Neurochemistry, 1972, Vol. 19, pp. 1483 to 1494. Pergamon Press. Printed in Great Britain.

THE INCORPORATION OF LABELLED ACETATE INTO CEREBROSIDE AND OTHER LIPIDS OF THE DEVELOPING MOUSE BRAIN

I. KANAZAWA, N. UEDA and T. YAMAKAWA

Department of Biochemistry, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan

(*Received* 7 *September* 1971. *Accepted* 30 *December* 1971)

Abstract—Cerebroside in the brain is highly localized in myelin and has a relatively slow turnover rate. The aim of this study was to evaluate the true cerebroside biosynthetic activity under conditions in which the degradation and reutilization of brain lipids were as small as possible. The 3-week-old mice were decapitated at 0·5, 1, 2·5, 5 and 15 min after the intraperitoneal injection of labelled acetate and the incorporation of radioactivity into each lipid class was examined. Even at 0·5 min, a considerable amount of radioactivity was found in simple lipids, especially in the free fatty acid fraction, and in the course of time the radioactivity of complex lipids increased. On the other hand, the incorporation of radioactivity into cerebrosides was extremely small throughout the experimental period. Results indicated that the low radioactivity of cerebroside might be due to its high content of long-chain fatty acids which were weakly labelled. The radioactivity of the sphingosine moiety was also low. In short, one of the rate-limiting steps of cerebroside synthesis in brain might exist in long-chain fatty acid and sphingosine synthesis. In addition, the incorporation curves of each component of cerebroside were compared with each other and the difference of the incorporation pattern of non-hydroxy fatty acids of cerebroside was noted.

MYELINATION is both a prominent and a unique morphological change in the developing brain. In accordance with the morphological development, the lipid composition of brain exhibits a significant change. FOLCH-PI (1955) showed that cerebroside was closely connected with the myelinating process and essentially similar results were obtained by UZMAN and RUMLEY (1958). BURTON, SODD and BRADY (1958) demonstrated that the incorporation *in vivo* of labelled hexose into brain cerebroside was a function of age, occurring most rapidly during the period of myelination. From these reports, it is clear that an increased rate of cerebroside occurs in the developing brain.

A group of investigators (HAJRA and RADIN, 1963*a*, *b*; HAJRA, KISHIMOTO, DAVIES and RADIN, 1965; KISHIMOTO and RADIN, 1965) demonstrated the incorporation of labelled acetate or fatty acid into brain cerebroside *in vivo*. In a series of studies, they clearly showed the mechanism of biosynthesis of cerebroside fatty acid. But their experiments were rather long-term studies and the accumulation of radioactivity in cerebroside may not reflect the pure biosynthetic activity of cerebroside because there must be reutilization of labelled compounds liberated from other lipids such as glycerophosphatides. It is therefore necessary to minimize such effects in experiments to evaluate the sole biosynthetic activity of cerebroside. In our experiments, mouse brain was removed at 0·5, 1, 2·5, 5 and 15 min after the intraperitoneal injection of labelled acetate and the incorporation into each lipid class of brain was followed. In contrast to the amount of radioactivity in glycerophosphatides, the radioactivity in cerebroside was very low even in the brains of 3-week-old mice when the cerebroside formation is thought to be most active.

MATERIALS AND METHODS

Experimental animals. Ten C3H/He mice of both sexes, 3 weeks of age, were obtained from the Institute of Medical Science of Tokyo University and used for this experiment.

Labelled compound. [1-^{14}C]Acetate 49·3 mCi/mmol, was purchased from Daiichi Kagaku Co. (Tokyo) and was used without further purification. Each mouse was injected intraperitoneally with 2 μl of an aqueous solution containing 0·05 mCi of [1-^{14}C]acetate, using a 10 μl microsyringe. At 0·5, 1, 2·5, 5 and 15 min, two mice were decapitated and their brains were immediately chilled with ice and kept at −20°C.

Extraction of lipids. Two brains of each group were triturated in a glass mortar with about 50 ml of chloroform–methanol (2 : 1, v/v) and kept for 24 h at room temperature for the complete extraction of lipids. The extract was filtered and the soluble fraction was obtained. The non-lipid components and most of the gangliosides were removed from this fraction by the method of FOLCH-PI, LEES and SLOANE-STANLEY (1957).

Separation of lipids. Separation of each lipid was performed by two-dimensional TLC (ROUSER, KRICHEVSKY, GALLI and HELLER, 1965). The silica gel plates, 20 × 20 cm, 0·3 mm thick, were prepared from Wakogel B-0 (Wako Pure Chem. Indust.) containing 10% magnesium silicate and activated at 120°C for 3 h before use. About 400 μg of total lipid was applied with a microsyringe for the separation. The plates were developed in the first dimension with the solvent mixture of chloroform–methanol–water–28% aqueous ammonia (130 : 50 : 8 : 0·5, by vol.) and the second dimension with chloroform–acetone–methanol–glacial acetic acid–water (100 : 40 : 30 : 20 : 12, by vol.). Lipid areas, detected with iodine vapour, were scraped off the plate after elimination of iodine and subjected to further analysis. Figure 1 shows the pattern of brain lipids separated on a two-dimensional TLC plate.

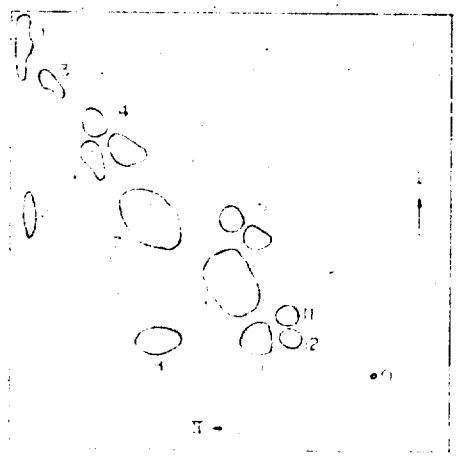


FIG. 1.—A two dimensional TLC of mouse brain total lipids. First development vertically (I) with chloroform–methanol–water–conc. ammonia (130 : 50 : 8 : 0·5, by vol.). After drying in a desiccator, second development to the left (II) with chloroform–acetone–methanol–glacial acetic acid–water (100 : 40 : 40 : 20 : 12, by vol.), iodine stain. The areas are: 0, origin; 1, cholesterol and other simple lipids; 2, free fatty acid; 3, unidentified glycolipid; 4, cerebrosides; 5, sulphatides; 6, cardiolipin; 7, phosphatidylethanolamine; 8, phosphatidic acid; 9, phosphatidylcholine; 10, phosphatidylserine; 11, sphingomyelin; 12, phosphatidylinositol.

Determination of radioactivities. As this study was short-term, it was important to eliminate any contaminating radioactive acetate that may have remained in each lipid fraction. This was achieved by adding 0·3 ml of unlabelled acetic acid to the lipid fraction in counting vials. The vials were then heated under an infrared light until the odour of acetic acid was lost. Counting was done in a Beckmann LS-133 liquid scintillation counter. The silica gel from the lipid areas of TLC were directly suspended in toluene containing 0·6% of DPO.

Quantification of lipids and lipid components. Cholesterol was determined by the method of ZAK (1957). Phospholipid phosphorus was determined by the method of BARTLETT (1959). Galactose in

the glycolipids was detected by the anthrone sulphuric acid method. Free fatty acid was measured by GLC using internal standards. Column condition was as described below. The non-hydroxy fatty acid methylester was examined by GLC. A coiled glass column, packed with 15% diethyleneglycol-succinate coated on Celite 545 HMDS was used with N_2 as the carrier gas. The hydroxy fatty acid methylester was determined as the *O*-trimethylsilyl derivative using the method described by YAMAKAWA, UETA and ISHIZUKA (1964).

Preparation of cerebroside. A sample of 20 mg of total lipids was dissolved in 0·5 ml of diethyl ether containing 5% acetic acid and applied to a 5 × 1·5 cm column of silicic acid. Simple lipids were eluted with 50 ml of diethyl ether and the polar lipid fraction was obtained with 50 ml of methanol. The latter fraction was evaporated to dryness and redissolved with a small volume of chloroform and applied to a 15 × 0·8 column of Florisil. After washing with 50 ml of chloroform, the glycolipid fraction was obtained with 75 ml of chloroform–methanol (2 : 1, v/v). This fraction was concentrated to a small volume for the preparative silica gel TLC. After development with chloroform–methanol–water (65 : 25 : 4, by vol.), the cerebroside bands (kerasine and phrenosine type of cerebroside) were scraped off the plate. The radioactivity of each cerebroside was measured on a small sample without extraction. The silica gel containing cerebroside was heated with 3 ml of 3% HCl–methanol at 100°C for 3 h in a screw-capped tube. The methanolysates of cerebroside were separated from the silica gel and applied to another TLC plate of Wakogel B-0 containing 10% of magnesium silicate for the separation of each cerebroside component. A two-step development system was used. The first solvent mixture, chloroform–methanol–water (65 : 25 : 4, by vol.), was allowed to run approx. 14 cm from the bottom of the plate. The plate was dried and then developed by the second solvent mixture, *n*-hexane–diethyl ether (85 : 15, v/v). The solvent was allowed to move 19 cm from the bottom of the plate. On this plate, methylgalactosides, sphingosine bases, hydroxy fatty acid methylesters and non-hydroxy fatty acid methylesters were separated in this order from the origin to the front (Fig. 2). The radioactivity in these components was determined by the method described above.

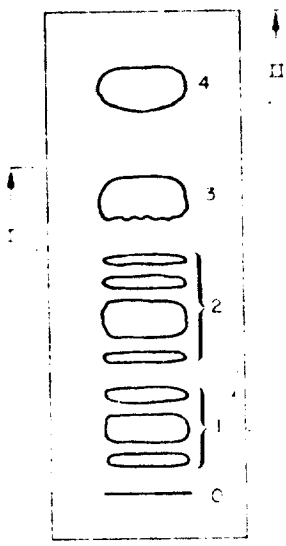


FIG. 2.—A two-step TLC of the methanolysates of brain cerebroside. First step development(I) with chloroform–methanol–water (65 : 25 : 4, by vol.). After drying in a desiccator, second development(II) to the same direction with *n*-hexane–ethyl ether (85 : 15, v/v), iodine stain. The areas are: 0, origin; 1, methyl-galactosides (anomers of ring isomers); 2, sphingosine and its methyl derivatives; 3, hydroxy fatty acid methylesters; 4, non-hydroxy fatty acid methylesters.

RESULTS

Most of the radioactivity administered intraperitoneally was lost as exhaled carbon dioxide within a few minutes after injection. The pattern of radioactivity of expiratory

carbon dioxide is shown in Fig. 3. From this curve, it is reasonable to conclude that the administered labelled acetate was readily absorbed from the peritoneal cavity and transferred to various organs, including brain, within a few minutes after injection. A considerable amount of radioactivity appeared in the brain lipid fraction as early as 0·5 min (Table 1). Within 5 min, a gradual increase in the percentage incorporation of radioactivity in brain lipids was observed. In Table 1, the percentage distribution of incorporated radioactivity in every lipid class of brain at the various times is shown. More than 60 per cent of the radioactivity of brain lipids was found in the simple lipid fraction at 0·5 min after injection, while less than 40 per cent was in the phospholipid fraction. The time course showed that the percentage distribution of radioactivity in the simple lipid fraction decreased and that of the phospholipid fraction increased markedly.

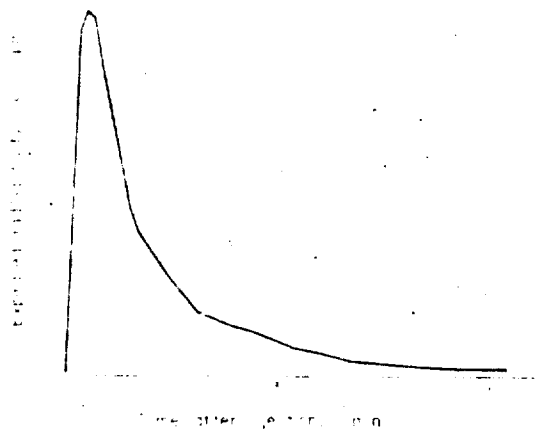


FIG. 3.—The expiration pattern of radioactive CO_2 after intraperitoneal injection of [1-^{14}C]acetate. After injection, the mouse was kept in a glass tube fitted with rubber stoppers in both sides. From one side of the tube, 30 ml of air per min was supplied and the other side of the tube was connected to thin-film window 4π gas flow counter and the radioactivity in the expired CO_2 was continuously recorded.

TABLE 1. THE PERCENTAGE INCORPORATION AND PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN BRAIN LIPIDS AND LIPID CLASSES AFTER THE INTRAPERITONEAL INJECTION OF [1-^{14}C]ACETATE

Time after administration (min)	0·5	1	2·5	5	15
Per cent of radioactivity administered*	0·07	0·09	0·15	0·35	0·19
Simple lipid fraction†	60·7	53·9	51·5	41·2	29·1
Phospholipid fraction†	38·8	45·4	47·6	57·2	66·7
Glycolipid fraction†	0·5	0·7	0·9	1·6	4·2

* The percentage incorporation of radioactivity into total lipid of brain after administration of [1-^{14}C]acetate.

† The percentage distribution of radioactivity among three classes of brain lipids.

Although the simple lipid fraction consisted largely of free cholesterol, small amounts of free fatty acid, triglyceride and cholesterol ester were also found. The distribution of radioactivity among these lipids is shown in Table 2. The incorporation of radioactivity into the free fatty acid fraction was already very prominent within 1 min after injection. As for the phospholipids (Table 3), the specific activities of phosphatidic acid and phosphatidylinositol were already high within the initial 1 min as compared to other phospholipids. The amount of radioactivity incorporated

TABLE 2.—THE INCORPORATED RADIOACTIVITY AND THE SPECIFIC RADIOACTIVITY OF EACH SIMPLE LIPID OF DEVELOPING MOUSE BRAIN AFTER ADMINISTRATION OF [1-^{14}C]ACETATE

Time after administration (min)		0·5	1	2·5	5	15
Free cholesterol	6·22 mg	12,804 (2058)	16,665 (2679)	29,361 (4721)	78,214 (12,575)	23,165 (3725)
Triglyceride	—	7110 (—)	8199 (—)	14,484 (—)	29,385 (—)	17,401 (—)
Esterified cholesterol	0·25 mg	2660 (10,641)	903 (3611)	4138 (16,553)	1837 (7346)	672 (2687)
Free fatty acid	0·71 mg	25,201 (35,494)	27,558 (38,814)	33,732 (47,510)	45,289 (63,787)	17,115 (24,106)
Others	—	1834 (—)	1558 (—)	3771 (—)	5280 (—)	2376 (—)
Total simple lipids	—	49,613 (—)	54,882 (—)	85,487 (—)	160,005 (—)	60,731 (—)

Numbers following the name of lipids indicate the content of lipid per brain. In pairs of figures, the upper one indicates the incorporated radioactivity (c.p.m./brain) and the lower one in brackets indicates the specific activity (c.p.m./mg of lipid).

TABLE 3.—THE INCORPORATED RADIOACTIVITY AND THE SPECIFIC RADIOACTIVITY OF EACH PHOSPHOLIPID OF DEVELOPING MOUSE BRAIN AFTER THE ADMINISTRATION OF [1-^{14}C]ACETATE

Time after administration (min)		0·5	1	2·5	5	15
Phosphatidylcholine	5·59 mg	14,388 (2574)	21,202 (3793)	42,734 (7645)	129,621 (23,188)	89,435 (15,999)
Phosphatidylethanolamine	5·23 mg	2986 (571)	4813 (920)	12,085 (2311)	41,307 (7898)	24,768 (4736)
Phosphatidylserine	2·22 mg	2413 (1087)	2935 (1322)	4381 (1973)	9175 (4133)	4525 (2038)
Cardiolipin	1·34 mg	575 (429)	374 (279)	525 (392)	1610 (1204)	313 (234)
Sphingomyelin	1·33 mg	331 (249)	401 (301)	630 (473)	1601 (1203)	2763 (2077)
Phosphatidic acid	0·93 mg	6843 (7358)	8185 (8801)	8082 (8690)	14,368 (15,449)	5124 (5509)
Phosphatidylinositol	0·91 mg	4089 (4493)	8197 (9007)	10,671 (11,727)	25,239 (27,801)	12,352 (13,574)
Total phospholipids	17·56 mg	31,625 (1801)	46,107 (2626)	79,108 (4505)	222,980 (12,698)	139,279 (7932)

Numbers following the name of lipids indicate the content of lipid per brain. In pairs of figures, the upper one indicates the incorporated radioactivity (c.p.m./brain) and the lower one in brackets indicates the specific activity (c.p.m./mg of lipid).

into phosphatidylcholine was by far the largest among the phospholipids. These findings are compatible with those of previous reports (SUN and HORROCKS, 1969; ANSELL and HAWTHORNE, 1964). It is interesting to note the higher specific radioactivity of phosphatidylserine than that of phosphatidylethanolamine in such a short-term experiment.

The radioactivity incorporated into the glycolipid fraction was extremely small but the activity of cerebroside steadily increased throughout the experimental period (Table 4, Fig. 4), while that of sulphatide showed an almost negligible increase. The

TABLE 4.—THE INCORPORATED RADIOACTIVITY AND THE SPECIFIC RADIOACTIVITY OF EACH GLYCOLIPID OF DEVELOPING MOUSE BRAIN AFTER THE ADMINISTRATION OF [1-^{14}C]ACETATE

Time after administration (min)		0·5	1	2·5	5	15
Cerebroside	1·63 mg*	281 (172)	308 (189)	1022 (627)	5016 (3077)	6984 (4284)
Sulphatide	0·41 mg*	69 (169)	104 (255)	163 (397)	312 (762)	358 (892)
Unidentified	36 μg*	51 (—)	277 (—)	389 (—)	1022 (—)	1351 (—)
Total glycolipids		401 (—)	690 (—)	1533 (—)	6359 (—)	8692 (—)

In pairs of figures, the upper one indicates the incorporated radioactivity (c.p.m./brain) and the lower one in brackets indicates the specific radioactivity (c.p.m./mg of lipid).

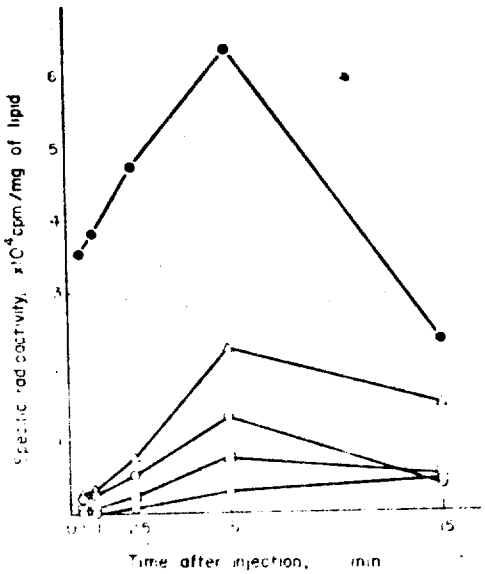
* Content of lipid per brain.

† Galactose value (determined by the anthrone method).

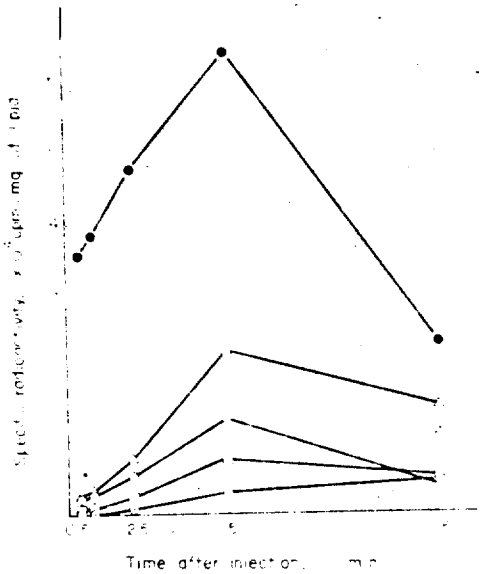
incorporation of radioactivity into each type of cerebroside (kerasine and phrenosine) was separately determined (Fig. 5). The incorporation of radioactivity into cerebroside components (galactose, sphingosine base and each type of fatty acid) is plotted in Fig. 6. A markedly different incorporation can be seen between the fatty acids. The radioactivity of non-hydroxy fatty acid was higher than of hydroxy fatty acid until after 2·5 min and remained unchanged thereafter. On the other hand, the radioactivity of hydroxy fatty acid was very low after 1 and 2·5 min and then increased rapidly, so the incorporation into non-hydroxy fatty acid was likely precedent to hydroxy fatty acid. The fatty acid pattern of brain cerebroside for this age of mouse is shown in Table 6. The incorporation of radioactivity into cerebroside sphingosine base was very low after 1 and 2·5 min and then increased rapidly like that of hydroxy fatty acid (Fig. 6).

DISCUSSION

Significance of a short-term experiment. With *in vivo* experiments, it is theoretically impossible to distinguish clearly between the biosynthesis and degradation of lipids. Considering the fact that brain lipids accumulate significantly in the mouse at this age (FOLCH-PI, 1955; UZMAN and RUMLEY, 1958), the activity of lipid formation must be much greater than that of degradation. Moreover, in a short term study, enough labelled acetate must be present in the brain and lipogenesis by re-utilization must be exceeded by the active incorporation of labelled acetate



(a)



(b)

FIG. 4.—Time course of incorporation of [1-^{14}C]acetate into several lipid classes of 21-day-old mouse brain. (b) Time course of specific activity of the same lipid classes as in (a).
△—phosphatidylcholine; ○—cholesterol; ●—free fatty acid; □—phosphatidylethanolamine; ◇—cerebroside.

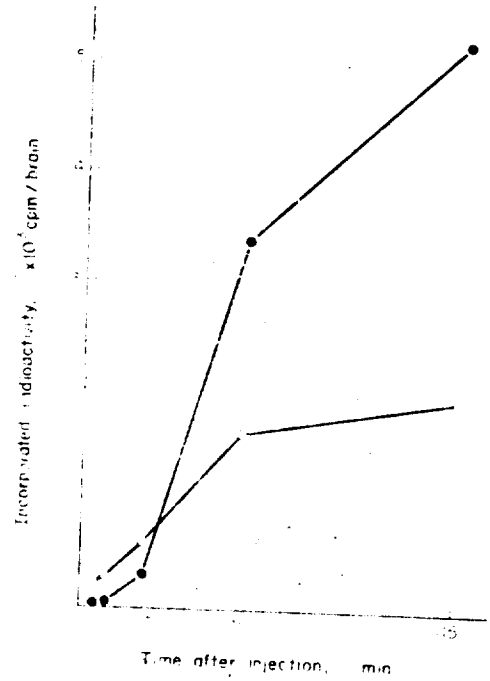


FIG. 5. Time course of incorporation of radioactivity into two types of cerebrosides. ● —phrenosine type of cerebroside; ○ —kerasine type of cerebroside.

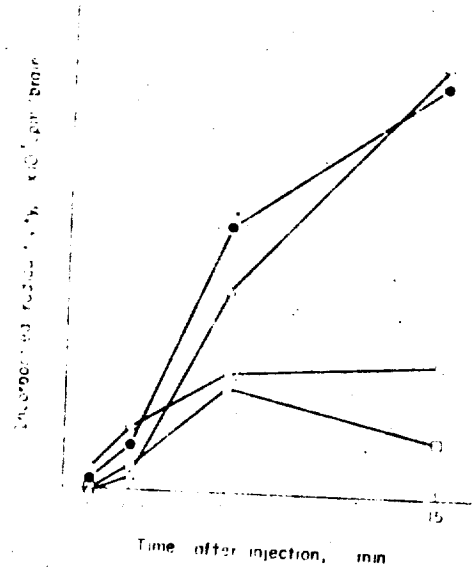


FIG. 6. Time course of incorporation of radioactivity into cerebroside components. ● —hydroxy fatty acid; ○ —non-hydroxy fatty acid; △ —sphingosine base; □ —galactose.

into lipids. Thus, the lipid degradation processes would seem to have a negligible effect on our results, especially within 2·5 min after injection. In this study the liquid nitrogen freezing method was not used because of the difficulty of taking the frozen brain completely out of the cranium. In a preliminary experiment, mouse heads were dropped into liquid nitrogen, instead of cooling in ice, with the same results as those described above.

Biosynthesis of simple lipid and phospholipid. In contrast to the previous long-term studies (MYANT and COLE, 1966; SUN and HORROCKS, 1969), more than 60 per cent of the radioactivity of brain lipids was found in the simple lipid fraction after 0·5 min, while the radioactivity in phospholipids was still low. This extremely rapid incorporation of radioactivity into simple lipids is largely due to the high amount of radioactivity in free fatty acid fraction. Using brain slices, LUNT and ROWE (1968) observed the incorporation of labelled acetate into free fatty acids. They demonstrated that the specific activity of free fatty acids was much greater than that of total esterified fatty acids in complex lipids. We also present evidence that the specific activity of free fatty acids formed *in vivo* is much greater than that of any other brain lipid. Although there still remains the possibility that a certain amount of free fatty acids are derived from the complex lipids, during tissue preparation and lipid extraction processes (WEBSTER, 1966; BAZAN and JOEL, 1970), the major part of free fatty acids might be expected to have arisen from *de novo* synthesis from acetate. BAZAN and JOEL (1970) reported that the amount of brain free fatty acids was about 0·04 per cent of the total lipids using the method devised by themselves. In contrast, the content of free fatty acids in our experiments was high, approx. 1·5 per cent of the total brain lipids and further study will be needed. But anyway, it is logical to propose that the labelled acetate is taken up into brain tissue and utilized for synthesis of free fatty acids at first and then the fatty acids are incorporated into complex lipids. Chain elongation, desaturation and hydroxylation processes should also occur in the course of fatty acid synthesis (KISHIMOTO and RADIN, 1965). In preliminary experiments, we found 30·4 per cent of $C_{16:0}$, 10·6 per cent of $C_{18:0}$, 13·6 per cent of $C_{18:0}$ ans other unidentified fatty acids in the free fatty acid fraction, but we could find no C_{24} acids. Therefore, there is no direct proof that these processes had occurred in the free fatty acid fraction of brain. Further study is needed to clarify this problem.

The incorporation curves of glycerophosphatides and sphingolipids were quite different from each other (Figs. 4 and 5). The former showed generally a steep rise in 5 min with a drop later, while the latter exhibited a slow and constant elevation even after 15 min. As for the glycerophosphatides, the up and down phase of the incorporation curves might express the active esterification and the hydrolytic processes.

Biosynthesis of brain cerebroside. Most of the brain cerebroside is located in myelin (O'BRIEN and SAMPSON, 1965). In mice of this age, myelin production is highly active and the cerebroside content increases very rapidly (FOLCH-PI, 1955; UZMAN and RUMLEY, 1958). Therefore, one would expect that the incorporation of radioactivity into cerebroside to be as high as that of other brain lipids. Contrary to this expectation, only a small amount of radioactivity was found in cerebroside, as shown in Fig. 4. However, the incorporation profile of acetate into cerebroside exhibited a constant rise throughout the experimental period as compared to glycerophosphatides. From this result, it can be assumed that the biosynthetic activity of cerebroside is low but the cerebroside, once formed, is comparatively stable. Consequently, though there is

TABLE 5.—THE INCORPORATED RADIOACTIVITY AND THE SPECIFIC RADIOACTIVITY OF TWO TYPES OF CEREBROSIDE AND CEREBROSIDE COMPONENTS OF DEVELOPING MOUSE BRAIN AFTER THE ADMINISTRATION OF [1-^{14}C]ACETATE

Time after administration (min)		0·5	1	2·5	5	15
Kerasine type of cerebroside	0·58 mg	220·8 (380·7)	250·0 (431·0)	627·4 (1081·7)	1706·4 (2942·1)	1940·0 (3344·8)
Phrenosine type of cerebroside	1·05 mg	59·9 (57·0)	58·3 (55·5)	394·2 (375·4)	3309·4 (3151·8)	5043·6 (4803·4)
Non-hydroxy fatty acid	0·23 mg	— (—)	177·6 (772·2)	417·8 (1816·5)	847·7 (3685·7)	909·3 (3953·5)
Hydroxy fatty acid	0·45 mg	— (—)	73·7 (163·8)	306·5 (681·1)	1888·4 (4196·4)	2789·9 (6199·8)
Sphingosine base	0·61 mg	— (—)	16·7 (27·4)	133·9 (219·5)	1452·1 (2380·5)	2930·3 (4803·8)
Galactose	0·34 mg	— (—)	40·3 (118·5)	163·3 (480·0)	827·6 (2434·1)	354·1 (1041·5)

Numbers following the name of lipids or lipid components indicate the contents per brain. In pairs of figures, the upper one indicates the incorporated radioactivity (c.p.m. brain) and the lower one indicates the specific radioactivity (c.p.m./mg of lipid or lipid component).

evidence that the breakdown of cerebroside occurs in young rat brain (KOPACZYK and RADIN, 1965) it accumulates. As shown in Table 5, most of the radioactivity incorporated into cerebroside was found in the fatty acid and sphingosine moieties. This seems reasonable in view of the lipogenetic pathway from acetate. The distribution of fatty acids in cerebroside (Table 6) shows a predominance of long chain fatty acids. KANAZAWA, UETA and YAMAKAWA (1970) have previously examined the incorporation

TABLE 6.—THE PERCENTAGE FATTY ACID COMPOSITION OF BRAIN CEREBROSIDE (MOUSE BRAIN, 3 WEEKS OF AGE)

	Non-hydroxy fatty acid	Hydroxy fatty acid
C 16 : 0	1·96	0·45
C 18 : 0	7·65	3·93
C 20 : 0	0·71	2·90
C 22 : 0	5·41	31·49
C 23 : 0	2·99	1·61
C 24 : 0	11·36	23·96
C 24 : 1	5·55	—
Total	35·60	64·40

of radioactivity into the total fatty acids of young mouse brain after intraperitoneal injection of labelled acetate. At 1 and 5 min after injection, respectively 87·5 and 92·9 per cent of total fatty acid radioactivity were found in the acids of C_{16-18} group. Furthermore, no radioactivity could be found in the acids of C_{24} group on radio-GLC. DHOPESHWARKAR, MAHER and MEAD (1969) reported essentially the same findings. From these data, it seems apparent that the low rate of biosynthesis of cerebroside must be due to the predominance of long chain fatty acids, which are weakly labelled. Some authors (BAUMANN, JACQUE, POLLET and HARPIN, 1968; BAUMANN-

HARPIN and BOURRE, 1970) presented evidence that in Quaking mouse brain the synthesis of long chain fatty acids might control the level of cerebroside. This would support our conclusion above.

The incorporation of radioactivity into sphingosine bases was small as was that of fatty acids (Table 5). KISHIMOTO, DAVIES and RADIN (1965) demonstrated in their long-term study that the incorporation of acetate into sphingosine and total fatty acids in cerebroside proceeded in a similar manner. As shown in Fig. 6, the incorporation profile of sphingosine was much the same with that of hydroxy fatty acid in our short-term experiment. It is not easy to interpret this finding. There are many reports on the *in vitro* biosynthetic pathway of brain cerebrosides but there is no established opinion as to whether psychosine or ceramide plays a role as the intermediate in cerebroside synthesis. MORELL, CONSTANTINO-CECCARINI and RADIN (1970) recently proposed that the rate-limiting steps of non-hydroxy fatty acyl cerebroside synthesis and of hydroxy fatty acyl cerebroside synthesis were different. Although it is difficult to compare directly the results of our *in vivo* experiments with those *in vitro* mentioned above, it might be reasonable to conclude from our data that the rate-limiting step or steps in cerebroside synthesis are possibly located in the synthesis of long chain fatty acids and sphingosine before they are incorporated into psychosine or ceramide. The incorporation of acetate into the hydroxy fatty acid of cerebroside, as shown in Fig. 6, exhibited an initial delay in rise in contrast to that of non-hydroxy fatty acid. BOWEN and RADIN (1968) discussed the possibility that the non-hydroxy fatty acid might be converted to its corresponding hydroxy fatty acid prior to its incorporation into cerebroside. The initial delay in the incorporation curve of hydroxy fatty acid in our results might possibly be interpreted from this hydroxylation process but further study will be needed.

REFERENCES

ANSELL G. B. and HAWTHORNE J. N. (1964) In *Phospholipids and the Nervous System*, Vol. 3, p. 278, BBA Library, Elsevier, Amsterdam.
BARTLETT G. R. (1959) *J. biol. Chem.* **141**, 738.
BAUMANN N. A., JACQUET C. M., POLLET S. A. and HARPIN M. L. (1968) *Eur. J. Biochem.* **4**, 340.
BAUMANN N. A., HARPIN M. L. and BOURRE J. M. (1970) *Nature, Lond.* **227**, 960.
BAZAN N. G., JR. and JOFE C. D. (1970) *J. Lipid Res.* **11**, 42.
BOWEN D. M. and RADIN N. S. (1968) *J. Lipid Res.* **6**, 255.
BURTON R. M., SODD M. A. and BRADY R. O. (1958) *J. biol. Chem.* **233**, 1053.
DOPESHWARKAR G. A., MAHR R. and MEAD J. (1969) *Biochim. biophys. Acta* **187**, 6.
FOLCH-PI J. (1955) In *Biochemistry of the Developing Nervous System* (Edited by WALSCH H.) p. 121. Academic Press, N.Y.
FOLCH-PI J., LEES M. and SLOANE-STANLEY G. H. (1957) *J. biol. Chem.* **226**, 497.
HAJRA A. M. and RADIN N. S. (1963*a*) *J. Lipid Res.* **4**, 270.
HAJRA A. M. and RADIN N. S. (1963*b*) *J. Lipid Res.* **4**, 448.
KANAZAWA I., UETA N. and YAMAKAWA T. (1970) *Bull. Jap. Neurochem. Soc.* **9** (*Suppl.*), 52 (In Japanese).
KISHIMOTO Y., DAVIES W. E. and RADIN N. S. (1965) *J. Lipid Res.* **6**, 525.
KISHIMOTO Y. and RADIN N. S. (1965) *Lipids* **6**, 47.
KOPACZYK K. C. and RADIN N. S. (1965) *J. Lipid Res.* **6**, 140.
LUNT G. G. and ROWE C. F. (1968) *Biochim. biophys. Acta* **152**, 681.
MORELL P., CONSTANTINO-CECCARINI E. and RADIN N. S. (1970) *Arch. biochem. Biophys.* **141**, 738.
MYANT N. B. and COLE L. A. (1966) *J. Neurochem.* **13**, 1299.
O'BRIEN J. and SAMPSON E. L. (1965) *J. Lipid Res.* **6**, 537.
ROUSER G., KRICHEVSKY G., GALLI C. and HELLER D. (1965) *J. Am. Oil Chem. Soc.* **42**, 215.
SUN G. Y. and HORROCKS L. A. (1969) *J. Neurochem.* **16**, 181.

UZMAN L. L. and RUMLEY M. K. (1958) *J. Neurochem.* **3**, 170.
WEBSTER G. R. (1966) *Biochem. J.* **98**, 19P.
YAMAKAWA T., UETA N. and ISHIZUKA I. (1964) *Japan. J. exp. Med.* **34**, 231.
ZAK B. (1957) *Amer. J. clin. Path.* **27**, 583.

THE EFFECT OF ACETYL DONORS ON CREATINE FORMATION IN RATS AND DOGS*

By ALEXANDER KANDEL and MAYNARD B. CHENOWETH

(*From the Department of Pharmacology, Medical School, University of Michigan, Ann Arbor, Michigan*)

(Received for publication, January 8, 1951)

Glycerol monoacetate has been employed in this laboratory as an effective antagonist to the toxic actions of sodium fluoroacetate in animals[1] (1). It has been assumed that such antagonism stems from the ability of glycerol monoacetate to provide labile 2-carbon chains to compete with fluoroacetate in the formation of one or more intermediary metabolites (2). An unusual extension of this ability to donate a metabolically active 2-carbon fragment was fortuitously discovered when it was observed that urine collected from dogs treated daily with glycerol monoacetate contained large amounts of creatinine and preformed creatine. The studies reported in this paper suggest a direct and active participation of glycerol monoacetate in the synthesis of glycocyamine *in vitro* and the formation of creatine *in vivo*. The synthesis of creatine and its congeners appeared, then, to be useful as a measure of the availability and activity of acetyl groups. Experiments were designed to demonstrate this acetyl donor property of glycerol monoacetate as well as to elucidate previous preliminary observations (3).

EXPERIMENTAL

In Vivo; Dogs—Urine was collected from unanesthetized female dogs, by means of a retention catheter, during 10 to 12 hour periods on separate days, the first of which constituted a control. Whenever possible, the same animals were used to compare glycerol monoacetate with other compounds. These were administered on different days after the control level of urinary creatine and creatinine was ascertained in each animal. The route of administration was intramuscular or oral. The compounds studied, with the exception of sodium acetate, were injected intramuscularly since vomiting occurred when they were given orally.

Creatinine was determined by a modification of the procedure employed by Bonanes and Taussky (4). Preformed creatine was converted to creatinine by boiling with N hydrochloric acid and determined by the difference between the total and preformed creatinine. Glycocyamine was determined by a modification of the Sakaguchi test (5), and an x-chromogen, probably glycocyamidine, by subtracting the value obtained for preformed creatinine by the dinitrobenzoate method (6) from that by the alkaline picrate method (4). Qualitative determinations were made for reducing substances, proteins, ketones, acetate, and lactate in the urine (7, 8).

In Vitro; Rats—Kidney cortex and liver slices and cell-free homogenates of renal cortex from Sprague-Dawley rats were used for these experiments. The method of Borsook and Dubnoff (9) was employed in studying glycocyamine and creatine formation in such preparations. All reagents and substrates were made up in Sørensen's phosphate buffer, pH 7.0. A Potter-Elvehjem homogenizer was used to macerate the kidney cortex. Only endogenous arginine was employed in these experiments. The reaction mixtures were incubated for 3 hours at 38° with 95 per cent O_2 and 5 per cent CO_2.

RESULTS AND DISCUSSION

The first two series of dogs (Series A and B, Table I) were treated with different samples of crude glycerol monoacetate assaying 70 and 56 per cent of the theoretical for the ester, respectively. Free acetic acid was removed by shaking the samples with alumina and filtering. The disparities in the total creatinine produced show some relation to the actual amounts of glycerol monoacetate employed. It is not known whether the α or β isomer of the ester, or what proportion of each, was present, nor which one was the active component. Glycerol monoacetate, however, did increase the urinary creatinine level, while other neutral acetyl donors[1] were, for various reasons of toxicity, unsuitable for extensive study, although some did occasion an increased excretion of creatine and creatinine.

Harrow *et al.* (10) found that acetate and glycerol increased the formation of acetylated *p*-aminobenzoate in rabbits. It was believed, therefore, that glycerol by a similar mechanism would contribute acetyl groups for the formation of creatine. In each dog examined, however, administration of glycerol produced no increase in the total creatinine. Although commercial samples of glycerol monoacetate contain fairly large amounts of glycerol, in view of the above findings it can be safely ventured that the increase in total creatinine in the urine reflects only the glycerol monoacetate content of the crude preparations.

Sodium acetate, given in amounts almost twice those of glycerol monoacetate, on an equimolar basis (see Table I), did not produce as marked an increase as the latter. In no animal receiving either glycerol monoacetate,

* This investigation was supported in part by a research grant from the National Heart Institute, National Institutes of Health, United States Public Health Service.

[1] Chenoweth, M. B., Kandel, A., Johnson, L. B., and Bennett, D. R., unpublished work.

glycerol, or sodium acetate was acetate detected in the urine. There was no evidence of reducing substances, proteins, or ketones. Lactates were found only in the urine of those animals which were injected with glycerol.

TABLE I

Comparison of Influence of Glycerol Monoacetate, Sodium Acetate, and Glycerol on Total Urinary Creatinine in Dogs

Average values of the number of animals indicated.

Series	No. of dogs	Total creatinine following				
		Control	Glycerol monoacetate*	Sodium acetate†	Glycerol†	Increase
		mg. per kg. per hr.	*mg. per kg. per hr.*	*mg. per kg. per hr.*	*mg. per kg. per hr.*	*per cent*
A	5	1.28 ± 0.05‡	1.65 ± 0.10‡			30
B	6	1.53 ± 0.06	1.84 ± 0.12			20
C	6	1.65 ± 0.12		1.91 ± 0.12‡		12
D	3	1.62 ± 0.06			1.60 ± 0.08‡	0

* Series A received 28 mM per kilo of glycerol monoacetate, and Series B 22 mM per kilo of glycerol monoacetate.
† 36 mM per kilo.
‡ Standard error.

TABLE II

Combined Effect of Glycerol Monoacetate and Guanidine on Total Urinary Creatinine in Dogs

Dog No.	Glycerol monoacetate	Guanidine	Glycocyamine	α-Chromogen (glycocyamidine)	Creatinine, total
	mM per kg.	*mM per kg.*	*mg. per kg. per hr.*	*mg. per kg. per hr.*	*mg. per kg. per hr.*
30					1.42
	28				1.64
		0.25*			1.42
	24	0.25*			1.31
	28	1.0†		0.28	1.12
52					1.11
	32	1.0‡	0.02	0.03	0.53

* Intravenously.
† Intramuscularly.
‡ Subcutaneously.

The appearance of glycocyamine following the simultaneous administration of guanidine hydrochloride, subcutaneously, and glycerol monoacetate, intramuscularly (Table II), could be related to the differential depression of the activities of the liver and the kidney cortex seen in the rat (Tables III and IV). In the dog, this depression could be observed only when the guanidine was administered subcutaneously in relatively large amounts.

The liver, the site of methylation of glycocyamine, is far more susceptible to the action of guanidine *in vitro* than is the kidney cortex, which probably accounts for the presence of glycocyamine and the decrease of total creatinine *in vivo*.

TABLE III

Depression of Glycocyamine Formation by Guanidine in Rat Kidney Cortex Slices

Guanidine added	Glycocyamine	
	Formed	Change
*mg. per gm.**	*mg. per gm.**	*per cent*
	0.36	
0.03	0.30	−19
3.00	0.29	−20
30.00	0.13	−64

* Calculated as wet weight of tissue.

TABLE IV

Depression of Creatine Formation by Guanidine in Rat Liver Slices

Glycocyamine added	Guanidine added	Creatine formed*
mg. per gm.†	*mg. per gm.†*	*mg. per gm.†*
		0.70
2.5		4.65
2.5	0.0003	2.10
2.5	0.03	1.15
2.5	0.3	0.50
2.5	3.0	0.00

* No creatinine formed in any experiments.
† Calculated as wet weight of tissue.

TABLE V

Influence of Glycerol Monoacetate and Sodium Acetate on Glycocyamine Formation in Rat Kidney Cortex Slices

Glycerol monoacetate	Sodium acetate	Glycocyamine			
		Formed		Change	
		Series A	Series B	Series A	Series B
M	*M*	*mg. per gm.**	*mg. per gm.**	*per cent*	*per cent*
		0.19	0.20		
0.002		0.35	0.32	+81	+46
0.004		0.35	0.48	+81	+123
	0.002	0.20	0.20	+3	−7
	0.004	0.30	0.33	+60	+53

* Calculated as wet weight of tissue.

397

On the basis of greater cellular permeation it was expected that glycerol monoacetate would surpass sodium acetate as a precursor of glycocyamine when both substrates were incubated with kidney cortex slices. This was shown to be true (Table V).

It seems rather unusual, however, to observe similar quantitative relationships existing when the two agents are incubated with a cell-free homogenate of the kidney cortex (Table VI). Diffusibility of non-polar compounds through the cell barriers appears not to be the only factor involved. Apparently, glycerol monoacetate provides a 2-carbon fragment which is either more active than sodium acetate or is more readily available at the site of acetylation.

TABLE VI

Influence of Glycerol Monoacetate and Sodium Acetate on Glycocyamine Formation in Rat Kidney Cortex Homogenate

Glycerol monoacetate	Sodium acetate	Glycocyamine			
		Formed		Change	
		Series A	Series B	Series A	Series B
M	*M*	*mg. per gm.**	*mg. per gm.**	*per cent*	*per cent*
		0.014	0.017		
0.002		0.020	0.029	+47	+65
0.004		0.022	0.035	+61	+102
	0.002	0.019	0.025	+40	+45
	0.004	0.019	0.025	+40	+45

* Calculated as wet weight of tissue.

SUMMARY

Glycerol monoacetate is more effective than sodium acetate in providing acetyl groups for the synthesis of creatine in the intact dog and in kidney cortex slices and homogenates of the rat. Glycerol is ineffective in this respect in dogs.

Guanidine depresses the methylating activity of the liver (as evidenced by creatine synthesis) to a greater extent than it does the acetylating activity of the kidney cortex of the rat. The effect of guanidine administration in the dog may be based on the type of activity of guanidine on the rat kidney cortex and liver *in vitro*.

BIBLIOGRAPHY

1. Chenoweth, M. B., Scott, E. B., and Seki, S. L., *Federation Proc.*, **8**, 280 (1949).
2. Chenoweth, M. B., *J. Pharmacol. and Exp. Therap.*, **97**, pt. 2, 383 (1949).
3. Kandel, A., and Chenoweth, M. B., *Federation Proc.*, **8**, 305 (1949).
4. Bonsnes, R. W., and Taussky, H. H., *J. Biol. Chem.*, **158**, 581 (1945).

5. Dubnoff, J. W., and Borsook, H., *J. Biol. Chem.*, **138**, 381 (1941).
6. Benedict, S. R., and Behre, J. A., *J. Biol. Chem.*, **114**, 515 (1936).
7. Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical physiological chemistry, Philadelphia, 12th edition (1947).
8. Feigl, F., Qualitative analysis by spot tests, New York, 3rd edition (1946).
9. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **138**, 389 (1941).
10. Harrow, B., Mazur, A., Borek, E., and Sherwin, C. P., *Biochem. Z.*, **293**, 302 (1937).

Res. vet. Sci., 1969, 10, 181

The Relationship between the Acidity of the Rumen Contents and Rumenitis, in Calves fed on Barley

M. KAY, B. F. FELL AND R. BOYNE
Rowett Research Institute, Bucksburn, Aberdeen

SUMMARY. Ayrshire calves were fed for 6 weeks on pelleted diets of barley that contained 7·5% of sodium bicarbonate or sodium acetate. The fall in pH of the rumen contents was less in these animals than in control calves fed on a similar diet to which neither bicarbonate nor acetate had been added.

At slaughter the lesions of hyperkeratosis, papillary clumping and rumenitis that occurred in the controls were reduced to negligible proportions in the calves fed on the experimental diet containing bicarbonate. The barley-acetate diet gave intermediate results with regard both to the pH of the rumen contents and to the severity of the ruminal lesions.

The essential differences between the rumens from calves fed on the 3 diets appeared to lie in the extent of mucosal thickening and folding. It is suggested that the pathological changes in the rumen wall, characteristically found in animals fed on barley, may begin with a thickening of the epithelium and lamina propria, and that this is a reaction to the fall in pH of the rumen contents. It is probable that the formation of mucosal rugae, due to epithelial and stromal proliferation, leads to the trapping of debris, and that the thickening shows further progression as the trapped hairs penetrate the epithelium and cause inflammatory reactions.

THIS INVESTIGATION WAS designed to clarify the part played by the pH of the rumen contents in the pathogenesis of rumenitis in calves fed on a diet containing 90% barley. Recent investigations made in calves during the transition from conventional feeding to a diet of barley have suggested that the pH of the rumen content does not fall below 5·0 during the first few weeks of barley feeding, and that it is within this period that the characteristic lesions develop in the ruminal epithelium (Fell, *et al.*, 1967; 1968).

There was some doubt in our minds as to whether pH 5·0 was sufficiently unphysiological to injure the ruminal epithelium, and the effects of acidity could not be dissociated from possible metabolic or pharmacological effects of the volatile fatty acids *per se*.

A preliminary feeding trial established that sufficient sodium bicarbonate to modify the fall in pH in the ruminal contents could be incorporated into the pelleted barley diet. Furthermore, the pattern of volatile fatty acid production typically found in animals fed on barley was not altered by the bicarbonate supplement. The results are now reported of further investigations into the effects of the modified diet. A diet of barley mixed with sodium acetate was also fed, so that possible effects of the sodium ion could be examined.

MATERIALS AND METHODS

Six Ayrshire bull calves were used in the investigation. They were reared to 90 kg. body weight in the way described by Fell *et al.* (1967). The calves were then moved into slatted-floor pens and were fed on a high-fibre change-over diet containing 5·10% crude fibre *ad lib.* for 2 to 3 days. The control diet and the 2 experimental diets were mixed with this feed and at the end of the third day the diets were fed *ad lib.* The calves were slaughtered 6 weeks after the change to the experimental diets.

Two calves were fed on each of the 3 diets, which were as follows.

The Barley Diet

The barley diet was regarded as the control diet, since the rumens from the calves fed on barley were to provide our standards for comparison. It was a pelleted diet containing 90% barley and 10% of a urea-mineral-vitamin mixture, as specified by Kay (1960). It had a crude protein content of 17·5% in dry matter.

The Experimental Diets

The 2 experimental diets were made by replacing barley in the barley diet with sufficient amounts of sodium bicarbonate or sodium acetate to give final concentrations of 7·5% additive.

Both the control diet and the barley-acetate diet had a calculated metabolizable energy content of 2·80 Mcal/kg. in the dry matter. The added acetate contributed approximately 5% to the total energy of the diet. The energy content of the bicarbonate diet was 5% lower than that of the other diets.

Analysis of the diets gave the following values for sodium:

Control diet	0·74%
Barley-acetate diet	2·53%
Barley-bicarbonate diet	2·58%

Determination of the Sodium Content of the Diets and of the Ruminal Liquor

An EEL flame photometer Model A* was used to determine the sodium content of the 3 diets and of specimens of ruminal liquor from calves eating the diets. The measurements were made on samples of the diets that were ashed and dissolved in 20% v/v conc. HCl, and on dilutions of the supernatant liquor obtained by the centrifugation of rumen samples.

Measurement of the pH of the Ruminal Liquor

Three times a week, at 9.30 a.m., samples of rumen contents were obtained by stomach tube and the pH of each specimen was recorded immediately using a Pye (Cambridge) pH meter.

The Estimation of the Volatile Fatty Acid Content of the Ruminal Liquor

Determinations of the total volatile fatty acid concentration in samples of ruminal liquor, and of the molar proportions of the fractions, were made by the methods used by Fell *et al.* (1968). For each calf, VFA estimations were done on one rumen sample obtained before the dietary change, and on one sample obtained during the fifth week after the change. The analyses were made to investigate the possibility that the bicarbonate and acetate additives had affected the patterns of ruminal fermentation.

Procedure at Slaughter

When the calves were slaughtered the volume of the rumen contents was recorded. The rumens were examined at the earliest opportunity and pieces of the wall of the ventral part of the anterior dorsal sac were weighed, placed in 10% formol-saline and set aside for dissection and histological examination. The rumens were washed, allowed to drain for 2 hr., and weighed.

At the end of the experiment, the preserved pieces of rumen wall were washed and blotted dry. The tissue was separated by blunt dissection into muscular and mucosal layers, and these were weighed separately so that the ratio of the weight of the muscular layer to that of the mucosal layer could be calculated. In addition, a standard surface area of muscle, measured from the serosal surface, was weighed, and the papillae in a standard area of mucosa were counted.

RESULTS

Food Consumption and Weight Gain

During the 6 weeks, the weekly weight gains and the daily food consumptions of the calves were recorded. The mean values of these measurements show a satisfactory standard of appetite and growth rate (Table I).

Volume of Rumen Contents at Slaughter

The calves on the high-sodium diets excreted considerably more urine than did the controls, and this was presumed to be a reflection of a greater water intake. It seemed possible that the increased fluid turnover would be associated with an increased volume of rumen contents. If this were true, it is conceivable that the pathogenic effects of the barley diet could be modified by the flushing away of sedimented food particles.

For these reasons, the volume of the rumen contents was recorded at slaughter, and the percentage of dry matter in the rumen contents was determined. The values so obtained, however, did not show significant dietary effects.

*Eel[illegible] Instruments Ltd., Harlow, Essex.

pH of the Ruminal Liquor

The results are given as a histogram in Fig. 1. The median values were:

control calves, pH 5·50 and 5·60.
barley-acetate diet, pH 5·85 and 5·85,
barley-bicarbonate diet, pH 6·00 and 6·35.

In specimens from each group of calves, the change in ruminal VFA concentration was associated with an increase in the molar proportions of propionic and butyric acids and with a fall in the proportion of acetic acid (Table II).

TABLE I

THE MEAN VALUES OF THE DAILY FOOD CONSUMPTION AND BODY WEIGHT GAIN OF 3 PAIRS OF AYRSHIRE CALVES FED EXPERIMENTAL DIETS FOR 6 WEEKS

Diet*	Number of calves	Food intake kg./day	Body weight gain kg./day
90% barley	2	3·84	0·74
82·5% barley, 7·5% sodium acetate	2	5·06	0·93
82·5% barley, 7·5% sodium bicarbonate	2	4·43	0·95

*All diets contained 10% urea-mineral-vitamin mixture.

TABLE II

CHANGES IN THE VOLATILE FATTY ACID COMPOSITION OF THE RUMEN LIQUOR IN 3 PAIRS OF AYRSHIRE CALVES, ASSOCIATED WITH THE CONSUMPTION OF EXPERIMENTAL DIETS. EACH FIGURE IS THE MEAN OF VALUES FROM 2 CALVES, OBTAINED (1) BEFORE THE INTRODUCTION OF THE EXPERIMENTAL DIET AND (2) 5 WEEKS LATER

Diet	VFA conc. m.eq./litre		Molar proportions % VFA: Acetic		Propionic		Butyric	
	1	2	1	2	1	2	1	2
90% barley	83·82	96·69	53·1	35·9	24·45	39·65	13·55	25·9
82·5% barley 7·5% sodium acetate	88·99	159·00	57·3	53·8	26·3	31·0	8·9	12·6
82·5% barley 7·5% sodium bicarbonate	92·99	122·65	55·1	38·0	26·1	37·1	9·76	18·09

Volatile Fatty Acid Composition of the Ruminal Liquor

The total VFA concentration of the ruminal liquor obtained after 5 weeks on the experimental diets was in all cases higher than that of the samples obtained before the dietary change.

Sodium Content of the Ruminal Liquor

The analysis of 6 or 7 samples of rumen contents from each calf showed that the dietary treatments did not have an appreciable effect on the sodium content of the ruminal liquor.

Macroscopic Appearance of the Rumen Wall

The walls of the rumens from the 2 calves fed on the control barley diet felt relatively stiff and hard when handled. The epithelium showed generalized hyperkeratosis, with a thickening and clubbing of papillae. The papillae appeared to be crowded together, thus giving a dense appearance to the mucosa. Ridges due to papillary fusion were present in the anterior dorsal sac of each rumen. In many parts of the rumens the papillae were bound into clumps that contained dry food particles and matted hair (Fig. 2). Most of the papillae were hard and opaque, but isolated, inflamed papillae occurred irregularly throughout the mucosae. In both calves the mucosa of the anterior pillars of the rumen showed ulcers that had raised edges; the non-ulcerated areas were hyperplastic. An encapsulated abscess was present on the serosal surface of one rumen in the region of the anterior pillar and in the other there were several enlarged lymph nodes on the serosal surface and in the deep tissues of the ruminal pillars. The dorsum of the rumen was in both cases chequered with dark areas in which the epithelium was necrotic and covered with adherent debris.

The appearance of the rumen was very different in the calves that were fed on the barley diet containing bicarbonate (Fig. 3). The rumen wall was soft and pliable and the papillae appeared to be longer and more widely spaced. The papillae in general were flat, as in conventionally-fed animals, and the combined effect of these differences was to give the mucosa a relatively open texture. A few papillae were slightly thickened, but in the main there were few signs of hyperkeratosis. There was slight clumping of papillae, but this was very much less than in the controls, and the clumps were moist and easily opened. A few hairs, which were easily displaced, lay between the papillae. The epithelium of the ruminal pillars was not hyperplastic,

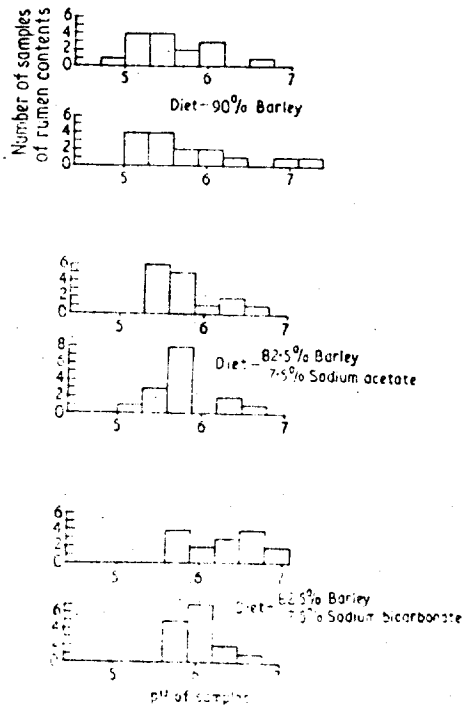


FIG. 1. Histogram showing the distribution of pH values in samples of rumen contents from 3 pairs of calves fed on all-concentrate diets. The observations were made between 9 and 42 days after the change from a conventional calf-rearing diet to diets of 90% barley, 82·5% barley–7·5% sodium acetate, and 82·5% barley–7·5% sodium bicarbonate.

and inflammatory changes were not seen in any part of the rumen wall.

In general, the barley diet supplemented with sodium acetate produced a condition of the ruminal mucosa intermediate between that of the other groups. In the calves fed on this diet the appearance of the mucosa, superficially, was similar to that found in the controls. Epithelial hyperkeratosis, clubbing and clumping of papillae were present, but in comparison with the situation in the calves fed on the control barley diet, the clumps of papillae were loosely formed and contained less sedi-

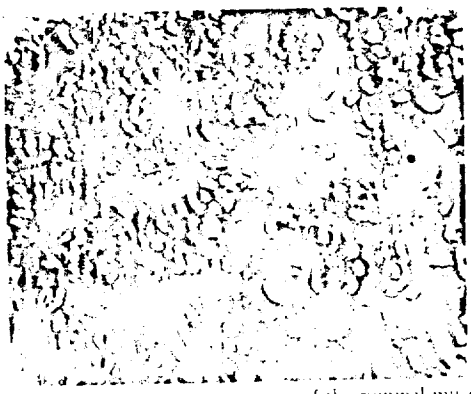


FIG. 2. The macroscopic appearance of the ruminal mucosa in the floor of the anterior dorsal sac, from a calf fed for 6 weeks on the 90% barley diet.

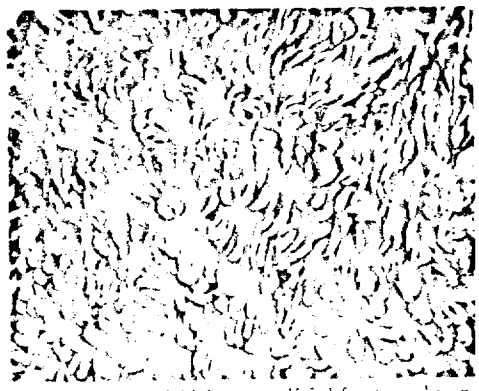


FIG. 3. A similar field from a calf fed for 6 weeks on the barley-sodium bicarbonate diet.

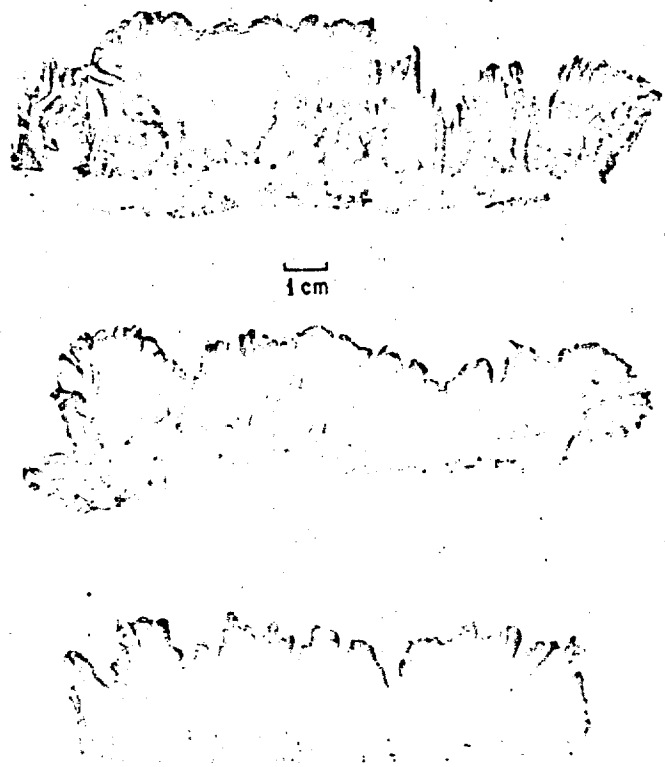
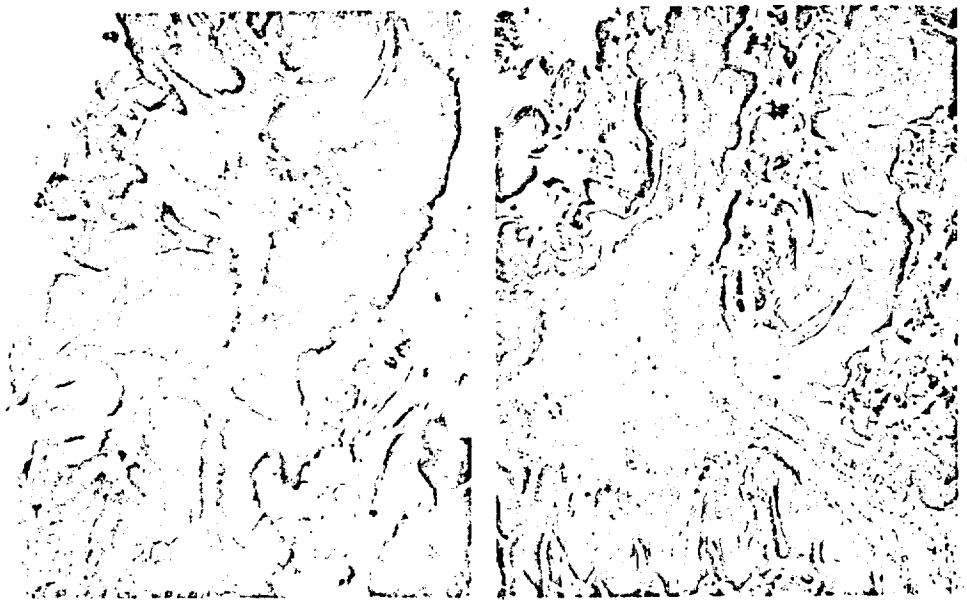
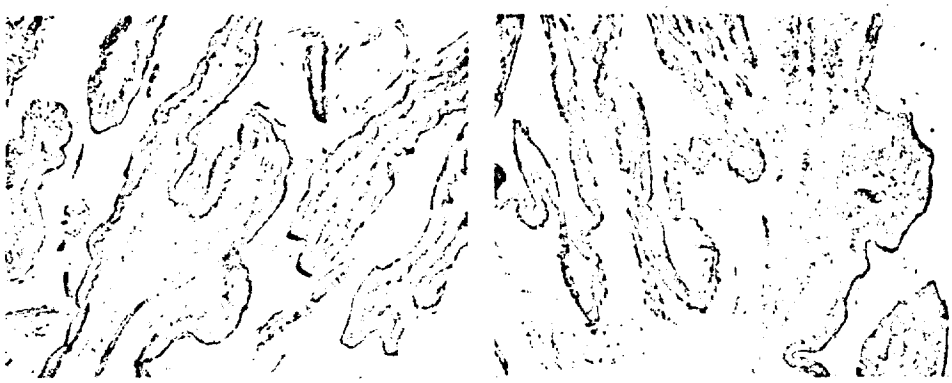


FIG. 4. Representative slices through the floor of the anterior dorsal sac of the rumen of calves fed for 6 weeks on the 3 diets: top, 90% barley; middle, 82·5% barley–7·5% sodium acetate; bottom, 82·5% barley–7·5% sodium bicarbonate. To illustrate the gradation in the extent of hyperkeratosis, papillary size and thickness of the lamina propria. Scale : 1 cm.



FIGS. 5 (left) & 6. Histological preparations of the ruminal mucosa from calves fed for 6 weeks on barley. Note the epithelial thickening and hyperkeratosis. H & E × 30.



FIGS. 7 (left) & 8. Similar preparations from calves fed on the barley-bicarbonate diet. The ruminal epithelium is relatively normal. H & E × 25.

mented material. Considerable amounts of hair were present, but the hairs were easily pulled out. Inflamed papillae were present irregularly throughout the mucosa, but no abscesses were found, neither was there enlargement of the regional lymph nodes.

Weight of the Rumen Wall

The weights of the rumens from the 3 pairs of calves were as follows:

Control barley diet:
5·73 and 5·77 kg.
Barley sodium acetate:
4·69 and 4·93 kg.
Barley-sodium bicarbonate:
4·22 and 4·82 kg.

Ratio of Muscular to Mucosal Weights

The mean values for the ratios of weight of muscle to weight of mucosa in pieces of the rumen wall from the floor of the anterior dorsal sac were as follows:

Barley diet	1:5·4
Barley-sodium acetate diet	1:3·9
Barley-sodium bicarbonate diet	1:3·5

There was little difference between the weights of standard areas of the muscular layer in the first and last groups, but the muscle was slightly heavier in the barley-acetate group.

Distribution and Size of Papillae

Barley Diet

The papillae were of various sizes, but generally were small, hard and club-shaped. The larger papillae were approximately 0·4 cm. long, the smaller ones were mere nodules. The papillae were counted in a square of mucosa 2 cm. × 2 cm. that was measured and cut out from below, after removing the muscular layer. It was difficult to make an accurate count of all the papillae because of the nodular structure of the smaller ones, but the counts for the 2 calves fell into the range 50 to 75 papillae/sq. cm.

Barley-Sodium Acetate Diet

The papillae were thickened and club-shaped, but less so than were similar specimens from the controls. Representative papillae measured 0·7 to 0·8 cm. The counts of papillae in the 2 calves ranged from 55 to 75/sq. cm.

Barley-Sodium Bicarbonate Diet

The papillae were soft and flat, and typically measured between 0·8 and 1·0 cm., although many smaller ones were present. There were between 50 and 70 papillae/sq. cm. of mucosa.

The Inter-Papillary Epithelium and Lamina Propria

The macroscopic examination of sections of the rumen wall, and of pieces of the de-papillated mucosa, revealed striking differences between the groups in the extent of the inter-papillary epithelium and in the amount of stroma present in the mucosa (Fig. 4). Large rugae were present in the ruminal mucosa of calves fed on the control barley diet. These folds did not involve the muscular layer of the rumen wall, but contained a fibrous core of lamina propria, so that overall this tissue was greatly increased in amount when compared with the specimens from calves that had received bicarbonate. Calves that had been fed on the barley diet supplemented with sodium acetate showed moderately severe folding and stromal proliferation.

Histological Appearance of the Rumen Wall

The ruminal mucosa of the control calves contained many lesions. These were consistent with our earlier experience of similar calves (Fell *et al.*, 1968). There were epithelial hyperplasia and hyperkeratosis (Figs. 5 and 6). The papillae were thickened, and there was a marked increase in the tissues of the lamina propria. The tips of some of

the papillae showed parakeratosis. Numerous inflammatory lesions due to the penetration of calf hairs were present in the epithelial and sub-epithelial tissues. Hairs were found commonly within intra-epithelial abscesses, and in fibrous foci within the lamina propria.

The rumens from the calves fed on the barley-bicarbonate diet were relatively normal (Figs. 7 and 8). Areas of hyperkeratosis were noted, but generally the papillae were slender and the tissues appeared to be free from parakeratosis, from intra-epithelial aggregations of leucocytes, and from stromal abscesses and fibrosis. The mucosa was relatively thin and there were no signs that any part of the tissues had been penetrated by foreign bodies.

The rumens from animals fed on the barley-acetate diet had a variable appearance. The epithelium was consistently hyperplastic, but otherwise some areas of the mucosa appeared to be normal, while adjacent areas showed hyperkeratosis, parakeratosis, acanthosis, and stromal thickening. Lesions due to the penetration of hairs were present, but these were much less frequent than in the controls.

DISCUSSION

The results of this investigation suggest that the fall in pH of the rumen contents in calves fed on barley plays an important part in initiating the lesions that develop in the ruminal mucosa. This inference is drawn from the beneficial effects obtained by the addition of sodium bicarbonate to the diet.

The interpretation of the results is complicated by the possibility that the increased fluid turnover that occurred in the calves fed on the barley-bicarbonate diet may have had a modifying influence on the pathological changes. The presence of moderately severe lesions in the animals fed on the barley-acetate diet, however, appears to be consistent with the view that low pH is the principal pathogenic agent, since the sodium intake of these animals was similar to that of calves fed on the barley-bicarbonate diet, but the pH of the rumen contents was lower.

It was pointed out by McGilliard *et al.* (1965) that VFA's in the rumen contents stimulate absorptive ability, metabolic activity and structural development. Undissociated volatile fatty acids penetrate the ruminal epithelium more rapidly than fatty acid anions, and consequently the penetration of VFA's through the ruminal epithelium is increased as the pH of the rumen contents falls. It seems probable that the thickening of the ruminal mucosa noted in calves fed on barley is due, initially, to the increased metabolic activity of the mucosa, brought about by the combined action of falling pH and rising VFA concentration. It would appear that under the extreme conditions associated with the consumption of the control barley diet the hypertrophic response of the ruminal mucosa may exceed the bounds of normality. Observations made during the dissection of specimens of rumen wall suggested that in the barley-fed control calves, in which the rumen contents had a median pH of 5·5 and 5·6, the mucosal thickening and folding, and the thickening and hardening of the papillae, had been sufficiently severe to firmly trap hairs and other debris, and that trapped hairs had subsequently penetrated the ruminal epithelium to cause secondary lesions and further thickening. In the barley-acetate group, in which the median pH of the rumen contents was 5·85, the mucosal rugae tended to be smaller and the papillae to be larger and softer than in the controls, and this marginal difference appears to have been sufficient to prevent the firm trapping of hairs, and

so to have greatly reduced the frequency of secondary inflammatory lesions.

The relatively normal appearance of the rumens from calves fed on the barley-sodium bicarbonate diet suggests, however, that concentration of VFA's similar to or higher than those recorded in the controls are not toxic *per se* when the median pH does not fall below 6·0.

Dobson and Phillipson (1956) found that the blood flow from the rumen in unanaesthetized sheep was increased by raising the concentration of VFA's in the rumen, and by lowering the pH of the rumen contents in the presence of VFA's. Lactate and phosphate buffers at pH 4·0 did not affect the blood flow. Thus it appears that the relationship between ruminal pH and VFA concentration on the one hand, and absorption, metabolic activity and structure on the other, may be dependent on the blood flow. The situation in the present investigation is complicated by the presence of superimposed inflammatory changes in the mucosa, but there was a definite correlation between the tendency to low pH in the rumen contents and the numbers of large blood vessels present in the lamina propria of the ruminal mucosa. This suggests that a falling pH in the rumen contents may lead to an expansion of the vascular bed.

ACKNOWLEDGMENTS

We thank Mr. Ian McDonald, of the Institute Biometry Department, for his statistical examination of the data, Mr. R. S. Reid for the determination of the volatile fatty acid contents of samples of rumen liquor, Mr. J. F. Ingram for the sodium assays and Mr. I. Robb for photographic work. We are grateful to the staff of the Duthie Experimental Stock Farm for their care of the animals.

Received for publication March 20th, 1968.

REFERENCES

FELL, B. F., BOYNE, R., KAY, M., WILLIAMS, R. B., & MACLEOD, N. A. (1967). *Vet. Rec.*, **81**, 593.

—— KAY, M., WHITELAW, F. G., & BOYNE, R. (1968). *Res. vet. Sci.*, **9**, 458.

DOBSON, A., & PHILLIPSON, A. T. (1956). *J. Physiol., Lond.* **133**, 76P.

MCGILLIARD, A. D., JACOBSON, N. L., & SUTTON, J. D. (1965). In 'Physiology of Digestion in the Ruminant'. Ed. R. W. Dougherty. pp. 39–50. London, Butterworths.

KAY, M. (1966). *Scott. Agric.*, **46**, 17.

The Conversion of Acetate to Cholesterol in the Fetus of the Baboon and the Transfer of Cholesterol from Mother to Fetus*

F. KHAMSI,† I. MERKATZ, AND S. SOLOMON

Departments of Biochemistry and Experimental Medicine, McGill University and the University Clinic, Royal Victoria Hospital, Montreal, Canada, and the Department of Obstetrics and Gynecology, Cornell University Medical College, New York

ABSTRACT. Tritium-labeled cholesterol was injected into two pregnant baboons in the third trimester of gestation and 5 days later laparotomy was performed and ^{14}C-acetate was injected into the umbilical vein and the circulation was intact for 110 and 119 min. In the first experiment the SA of the maternal ^{3}H-cholesterol (free and esterified) reached a constant level by the 5th day at which time there was complete equilibrium between the maternal and fetal free, but not the esterified, cholesterol. The study of the SA of ^{14}C- and ^{3}H-cholesterol of various tissues indicated that the placenta actively incorporated the maternal cholesterol but did not show any *de novo* synthesis of cholesterol. The fetal liver was a major site of synthesis of cholesterol whereas the synthesis and incorporation of cholesterol in the brain was small. None of the several neutral steroids searched for could be isolated from the liver in the second experiment and from the adrenals in both studies, indicating that the conversion of acetate and cholesterol to neutral steroids may be extremely small and undetectable by the experimental design employed for the study of these tissues. (*Endocrinology* **91:** 6, 1972)

THE PREGNANT baboon has been the subject of investigations as a possible model for human pregnancy and available knowledge indicates many similarities in endocrine function between the baboon and man. In the pregnant baboon there is an increasing production of estrogens with advancing pregnancy and an abrupt fall at the time of parturition as in man. However, the main urinary estrogen in the baboon is estrone rather than estriol as in man. In addition, the amount of estrone excreted in the baboon (100–200 μg/24 hr) is much less than estriol found in human pregnancy (1). Although pregnanediol was readily detected in the urine of the pregnant baboon in the last two months of pregnancy (100 μg/24 hr) it is much less than the amount excreted in human pregnancy urine (1,2). In our laboratory, studies in the pregnant baboon have been conducted by injecting labeled steroids into the umbilical vein and isolating the metabolites from the placenta and the fetal tissues (3). In one experiment, 7-^{3}H-pregnenolone and 4-^{14}C-dehydroisoandrosterone were injected into the umbilical vein and it was shown that pregnenolone can be hydroxylated by the fetal adrenal at the 11β-, 17α- and 21- positions as well as being metabolized to C_{19} steroids. Pregnenolone was not converted to C_{19} steroids in the placenta, whereas, this tissue converted dehydroisoandrosterone to estrogens, a situation analogous to that in man. In another study, when ^{3}H-progesterone and ^{14}C-cholesterol were injected into the umbilical circulation, it was shown that the fetal adrenal was capable of forming cortisol from progesterone, but, that cholesterol was not metabolized to neutral steroids. In the human mid-term fetus a very small conversion of cholesterol to neutral steroids has been demonstrated in the adrenals and perfusate with the use of perfusion techniques (4–6).

The aim of the present study was to assess the transfer of maternal cholesterol into the placenta and fetal tissues of the baboon and to compare this with *de novo* synthesis of cholesterol from acetate in the same tissues. Also, the formation of neutral steroids from acetate and cholesterol was investi-

Received September 2, 1971.

*The investigations reported in this paper were supported by Grants from the US Public Health Service (HDO-4365) and the Medical Research Council of Canada (MT-1656).

†Fellow of the Medical Research Council of Canada.

gated. In the human it has been shown that the maternal cholesterol is the main substrate for the formation of progesterone by the placenta (7,8) and it also contributes to a varying degree to the cholesterol present in the various fetal tissues (8–10).

Materials and Methods

Celite and alumina column chromatography, the determination of infrared spectra, and the measurement of radioactivity employed in these studies have been described previously (11,12). The solvent systems used for paper and thin-layer chromatography are described in Table 1.

Experimental animals

Two pregnant baboons were used for these experiments. They were housed at the Laboratory for Experimental Medicine and Surgery in Primates in Sterling Forest, New York, where the surgery was also carried out.

The first study was performed on a baboon who weighed 14 kg and was in the 168th day of gestation. At Day 1 of the study she was given an iv injection of 1.7×10^9 dpm of 7-^{3}H-cholesterol and daily blood samples (6 ml) were taken. On the 6th day the baboon was anesthetized by an initial im injection of Phencyclicine (Sernylan) and thereafter was maintained on a mixture of nitrous oxide and oxygen. Laparotomy was performed and a loop of pulsating umbilical cord was exposed through a window in the uterine wall. A solution containing 3.26×10^9 dpm of 1-^{14}C-acetate was injected slowly into the umbilical vein over a period of 5 min via a small scalp vein catheter. The umbilical circulation was intact with the fetus *in utero* for 119 min during which time the fetus died. Subsequently, the cord was clamped and the fetus and placenta were delivered. The female fetus had a crown-rump length of 22.5 cm and weighed 823 g. Various fetal tissues were dissected and frozen for transportation. The baboon was well and a further blood sample was taken 5 days after the operation.

The second study was performed in essentially the same manner, with a total of 9.35×10^8 dpm of 7-^{3}H-cholesterol injected iv on Day 1 and two samples of blood taken from the mother on Day 5, one at 9:30 AM at the start of surgery and one at 12:30 PM, when the operation was finished. A total of 2.16×10^9 dpm of 1-^{14}C-acetate was injected into the umbilical vein at 10:23 AM and the fetus and placenta were delivered 110 min later. The fetus was alive during the whole of this period. The baboon weighed 13.0 kg and was at the 160th day of gestation, and the female fetus weighed 700 g.

TABLE 1. Solvent systems used in chromatography

	System	Solvents
Paper chromatography	A	ligroin B:methanol:water (10:9:1)
	B	benzene:cyclohexane (1:1):propylene glycol
	C	ligroin C:methanol:water (100:75:25)
	D	ligroin C:propylene glycol
	E	benzene:cyclohexane (1:1):propylene glycol
	F	benzene:methanol:water (2:1:1)
	G	skelly C:benzene:methanol:water (12:0:50:8:2:0)
	H	skelly C:benzene:methanol:water (20:1:16:4)
Thin-layer chromatography	I	heptane:ethanol (95:5)
	J	ethyl ether:benzene:ethanol:acetic acid (40:5:2:2) redevelopment in ether:hexane (1:99)
	K	benzene:ethyl acetate (3:1)
	L	cyclohexane:ethanol (95:5)
	M	chloroform:acetone (95:5)

Extraction of tissues

The procedures have been described previously (4). In summary, the tissues were homogenized, extracted by ethanol, acetone (3:2), followed by 80% ethanol and partitioned between *n*-hexane and 90% methanol. The 90% methanol extract was partitioned between ether and water.

Labeled substrates

The 1-^{14}C-acetate and 7-^{3}H-cholesterol were purchased from New England Nuclear and the acetate was administered without purification. The ^{3}H-cholesterol was purified by chromatography in systems J and K in Experiment 1 and in three systems in Experiment 2, the final being system I. In both instances, an aliquot of the purified ^{3}H-cholesterol was mixed with carrier and the mixture was crystallized to constant SA. From these data it was possible to calculate that the ^{3}H-cholesterol was at least 95% pure.

Analysis of cholesterol

All the blood samples analyzed were in the form of plasma except for the fetal blood in the second experiment in which whole blood had to be used due to marked hemolysis. The isolation of blood cholesterol was carried out as described by Hellig *et al.* (8). The free and ester cholesterol were obtained by means of Florisil Column

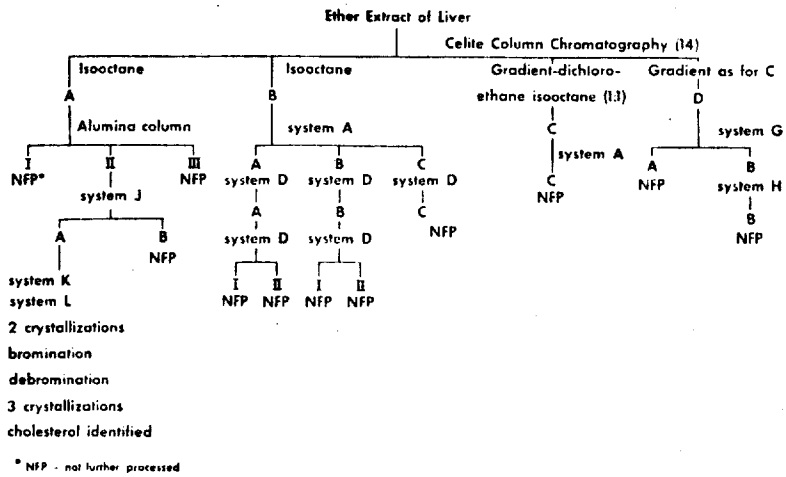


FIG. 1. Outline of the procedure used in the purification of the ether extract of the liver, Experiment 2.

chromatography of the extract using the method of Carroll (13).

The hexane extracts of the liver, Experiment 1, brain, Experiments 1 and 2, and placenta, Experiments 1 and 2, were subjected to column chromatography on Florisil. The same method of separation described above was used for these extracts.

In Fig. 1 is shown an outline of the procedures employed in the fractionation of the ether extract of the liver from Experiment 2 using Celite column chromatography as described by Engel *et al.* (14). As the ether extract was processed before the hexane fraction and because there was a sufficient weight of cholesterol for isolation, the hexane fraction was not further investigated. The free cholesterol was eluted in fraction A of the Celite column described in Fig. 1 and it was purified by chromatography, crystallization, and formation of the dibromide (15). The infrared spectrum of the isolated material was identical with that of standard cholesterol.

After chromatography, all the fractions of cholesterol were subjected to crystallization, bromination and debromination with subsequent crystallization. When the mass was small, carrier cholesterol was added and the same purification procedure was employed as will be described subsequently. In the case of the liver, brain and placenta in both Experiments 1 and 2, sufficient wt was available to determine infrared spectra which were identical with that of the standard cholesterol.

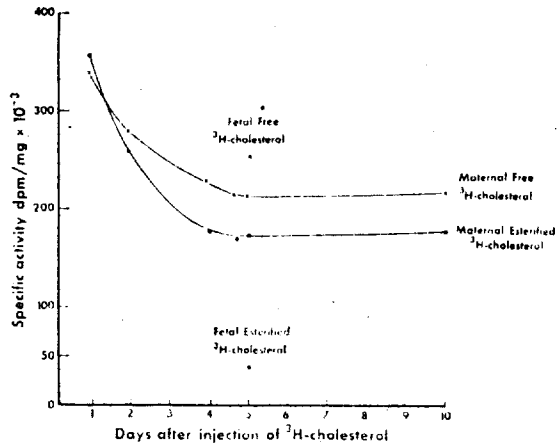


FIG. 2. SA of maternal and fetal plasma free and esterified cholesterol. Experiment 1.

Results

Circulating cholesterol

Fig. 2 illustrates the change in specific activity of maternal plasma ^{3}H-cholesterol in Experiment 1. A constant SA was obtained by the 5th day. The SA of fetal free ^{3}H-cholesterol was almost the same as the maternal value, whereas, the SA of fetal esterified ^{3}H-cholesterol was much lower.

The plasma free cholesterol fractions in Experiment 1 of Days 1, 2, 4 and 5 (PM) and that from the fetus were pooled after their SA were determined. Carrier cholesterol was added, the mixture was crystallized followed by bromination and debromination and a further three crystallizations as outlined in Table 2. The data obtained indicate that before carrier was added, radiochemi-

TABLE 2. Proof of radiochemical purity of cholesterol isolated from maternal plasma, experiment 1

	SA—dpm/mg			
	Crystals		Mother liquors	
	^{3}H	^{14}C	^{3}H	^{14}C
Prior to bromination	10840	100	10980	97
After bromination 1.	10830	100	11080	99
2.	11000	82	11100	110
3.	11200	110	11180	100
Calculated values*	11820	100		

* The calculated values were obtained by dividing the total radioactivity, 85197 dpm ^{3}H and 813 dpm ^{14}C by the total wt 7.208 mg. The wt of pooled free cholesterol fraction was 1.197 mg to which 6.011 mg carrier cholesterol was added.

cally pure cholesterol was obtained. The same procedure was carried out for the esterified fractions and the same conclusions reached. The free and esterified cholesterol fractions from Days 5 (AM) and 10 were each processed separately in the same manner and were shown to have been radiochemically pure before the addition of carrier cholesterol. The SA of cholesterol formed from acetate injected into the fetus in Experiment 1 was very low in both the fetal and maternal circulation.

In Table 3 is shown the SA of maternal and fetal cholesterol isolated on Day 5 of Experiment 2. The specific activity of the free ^{3}H-cholesterol in two samples of the maternal plasma, taken 3 hr apart, was constant but higher than the values obtained from fetal blood. The two maternal samples were pooled, brominated and debrominated, subjected to thin-layer chromatography in system J, and the SA determined on the material eluted from the plate was 64,730 dpm/mg indicating that the cholesterol was at least 90% pure. Table 3 also shows the SA of free ^{14}C-cholesterol (derived from the ^{14}C-acetate injected into the umbilical vein) which was much higher in the fetal blood compared to the maternal plasma.

Tissue cholesterol

Table 3 shows the SA of free cholesterol isolated from placenta, liver and brain. Table 4 shows an example of crystallization data, from a tissue such as the placenta in Experiment 1.

In Table 3 a comparison is made of the SA of free cholesterol isolated from various tissues in Experiments 1 and 2. The SA of the free ^{3}H-cholesterol is low in the brain and high in the other tissues, and the SA of free ^{14}C-cholesterol is low in the brain, placenta and maternal plasma but high in the liver. The SA of ^{14}C-cholesterol was low in the fetal plasma in Experiment 1 and high in fetal whole blood in Experiment 2.

Ether fraction of liver

This fraction weighed 276 mg and was chromatographed on a 200-g Celite column. Fig. 1 shows the various fractions eluted from the column which were subsequently

TABLE 3. Specific activity of tissue free cholesterol following injection of ^{3}H-cholesterol into the maternal circulation and ^{14}C-acetate into the umbilical vein in experiments 1 and 2

	Experiment 1 SA—dpm/mg			Experiment 2 SA—dpm/mg		
Tissue	^{3}H	^{14}C	100 × $^{14}C/^{3}H$	^{3}H	^{14}C	100 × $^{14}C/^{3}H$
Maternal plasma	210540	5120	2.4	74090 (9:30 AM) 70810 (12:30 PM) 64730 (pooled)	650	1.0
Fetal plasma	252480	2790	1.1			
Fetal whole blood				43510	26800	61.6
Placenta	82210	510	0.6	83010	1100	1.3
Fetal liver	18800	31640	168	33090	50560	152.8
Fetal brain	500	790	157	457	1880	412.0

TABLE 4. Proof of radiochemical purity of cholesterol isolated from the placenta in experiment 1

	Crystallization	SA—dpm/mg Crystals ^{3}H	Crystals ^{14}C	Mother liquors ^{3}H	Mother liquors ^{14}C
Initial value	1	28710	320	28500	180
After bromination and debromination	1	28500	350	28590	340
	2	28810	340	29020	340
	3	29010	370	28010	330

subjected to paper and thin-layer chromatography. From the previous experience with this type of column, the fractions known to contain pregnanolone[1], pregnenolone, progesterone, pregnanediol, dehydroisoandrosterone and testosterone were eluted and submitted to paper and thin-layer chromatography. Wherever there was enough radioactivity, carrier was added and the mixture was crystallized, the radioactivity partitioned into the mother liquors and in this manner it was possible to rule out the presence of the above neutral steroids.

Extracts of adrenals

The ether extract of the adrenals in Experiment 1 weighed 3.01 mg and contained 1711 dpm of ^{3}H-labeled and 7065 dpm of ^{14}C-labeled material and in Experiment 2 they weighed 3.2 mg and contained 3007 dpm of ^{3}H and 118,453 dpm of ^{14}C. As the weights of these two extracts were small, they were subjected to paper chromatography in system A. Since there were no distinct radioactive peaks when the paper was scanned, the areas corresponding in mobility to standard pregnenolone, progesterone, dehydroisoandrosterone, cortisol and cortisone were eluted. The amount of radioactivity in each eluate was determined, and where adequate amounts of radioactivity were found the fraction was subjected to further chromatography. By this means and by the addition of carrier to appropriate fractions followed by crystallization the presence of pregnenolone, progesterone, dehydroisoandrosterone, cortisol and cortisone was excluded.

The "sulfate" of pregnenolone, progesterone, dehydroisoandrosterone, cortisol and cortisone were also looked for but could not be detected.

[1] Pregnanolone: 5β-pregnan-3α-ol-20-one; pregnenolone: 3β-hydroxy-5-pregnen-20-one; pregnanediol: 5β-pregnan-3α,20α-diol.

Discussion

In Experiment 1, when serial blood samples were taken after the injection of ^{3}H-labeled cholesterol into the maternal circulation, the SA of both free and ester cholesterol fell rapidly until the 5th day, but remained constant until the last specimen was taken at the 10th day. These results are in agreement with experiments performed on non-pregnant adult baboons by Shapiro *et al.* (16). In Experiment 2, similar serial blood estimations were not available but two specimens taken on the 5th day after the injection of cholesterol had a constant SA (Table 3).

As illustrated in Table 3, 5 days after the injection of ^{3}H-cholesterol into the maternal circulation in Experiment 1, the SA of fetal plasma free ^{3}H-cholesterol was slightly higher than the maternal value (252,480 and 210,540 dpm/mg respectively). In Experiment 2, the SA of free ^{3}H-cholesterol in fetal blood was 0.7 of the maternal value (Table 3). This indicates that in the pregnant baboon, at the third trimester, maternal blood free cholesterol readily enters the fetus. However, in the human the passage of free cholesterol from the maternal into the fetal blood might be less (8) and it might vary according to the time of gestation when the study is carried out (10).

In Experiment 1, when both free and esterified cholesterol were measured it was demonstrated that the SA of esterified ^{3}H-cholesterol was much less in the fetal than in the maternal plasma. Two possibilities can be advanced to explain this data: either the placenta prevented transfer of the maternal esterified cholesterol into the fetus, or the maternal free cholesterol was esteri-

fied much less by the fetus than it is in the maternal compartment.

With respect to the SA of ^{14}C-cholesterol derived from ^{14}C-acetate in the fetal and maternal blood, there is a difference between the two experiments. The higher specific activity of ^{14}C-cholesterol in the fetal blood of the second experiment as compared to the first one may be due to a difference in the time of survival of the two fetuses, as the fetus in Experiment 1 died approximately 1 hr after the injection, whereas the fetus in Experiment 2 was delivered viable 110 min after the injection. Since ^{14}C-acetate injected into the umbilical vein crosses the placenta, ^{14}C-cholesterol in the mother may be due to *de novo* synthesis in the mother and this makes it difficult to make suggestions with respect to the transfer of fetal ^{14}C-cholesterol to the maternal side.

From the results of both experiments (Table 3) it is possible to conclude that very little cholesterol is made in the placenta from ^{14}C-acetate. The data obtained from the *in vitro* and *in vivo* experiments have yielded somewhat conflicting conclusions. *In vitro* it has been shown that the placenta possesses enzymes for the conversion of acetate to cholesterol (17). From their *in vivo* studies, Davis *et al.* (9) concluded that *de novo* synthesis of cholesterol from acetate by the placenta had occurred. However, Hellig *et al.* (8) interpreted their results to indicate that most of the cholesterol in the placenta was derived from the maternal blood cholesterol. When human placenta was perfused in a chamber with radioactive acetate, only a very small conversion of acetate to cholesterol was demonstrated (6). The results obtained in Experiments 1 and 2 are in agreement with this conclusion. In spite of low *in situ* synthesis of cholesterol from precursors, the placenta contains a large quantity of free cholesterol (283 and 181 mg in Experiments 1 and 2 respectively, uncorrected for losses during purification procedures) and the SA of free ^{3}H-cholesterol was high indicating that the placental cholesterol is probably derived from maternal sources. In Experiment 2 as opposed to Experiment 1, the SA of free ^{3}H-cholesterol is higher in the placenta than in the maternal blood and it is difficult to forward an explanation for this. In both experiments the SA of free ^{14}C-cholesterol in the fetal liver derived from acetate injected into the fetus was comparatively high (Table 3) indicating that this organ is a major site of formation of cholesterol in the fetus. Similar conclusions were also reached from studies on the adult baboon (7) and the human fetus (8–10).

The results obtained from the study of the brain are different from other tissues. In this tissue large quantities of free cholesterol were isolated (675 mg in Experiment 1 and 436 mg in Experiment 2) but their SA were very low with respect to both isotopes. Similar findings have been previously reported by others. Plotz *et al.* (9) administered ^{3}H-cholesterol orally to pregnant women for 15 days prior to therapeutic abortion, and 2–4 hr before the operation an injection of ^{14}C-acetate was given. The SA of cholesterol derived from either of the isotopes was very low in the brain as compared to other tissues. Similar results were obtained by Kabara and Werthessen (7) who administered ^{14}C-acetate to adult baboons and found the SA of brain ^{14}C-cholesterol to be considerably lower than other tissues. In neither of the two investigations discussed above were blood cholesterol levels determined, and therefore, the possibility exists that the radioactivity in the brain cholesterol might be derived from the cholesterol in the blood. The results from both experiments in our study militate against this interpretation. In Experiment 1 the SA of free ^{3}H-cholesterol was 252,480 dpm/mg in the fetal plasma and 502 dpm/mg in the brain, *i.e.*, only 0.002 of the fetal plasma cholesterol was incorporated into the brain cholesterol. This implies that the contribution of fetal free ^{14}C-cholesterol to the brain would be 2790 × 0.002, *i.e.*, 5.5 dpm/mg. However, the SA of brain free ^{14}C-cholesterol was 790 dpm/mg indicating that this was almost entirely due to *de novo* synthesis

of cholesterol from ^{14}C-acetate. The same explanation can be advanced for the data obtained in Experiment 2.

The low SA of the brain cholesterol can be interpreted either as low rate of synthesis and incorporation of cholesterol or it is due to dilution of the radioactive cholesterol in a large quantity of non-radioactive cholesterol present in the brain. The former explanation is supported by the fact that the total radioactivity in the brain hexane fraction was much less than that found in the liver (*e.g.*, 249,000 dpm ^{3}H in the brain and 2,238,000 dpm ^{3}H in the liver in Experiment 1).

A search was made for neutral steroids and their metabolites in the ether extract of the adrenals, the liver of Experiment 2 and the aqueous extract of the adrenal of Experiment 1. None of the several neutral steroids which were searched for were found. Similar results were obtained by Leung, Merkatz and Solomon (unpublished data) who injected ^{14}C-cholesterol into the umbilical vein of a baboon and were unable to detect any neutral steroids.

In conclusion, it appears that there are many similarities between the two members of the primate order, man and baboon, with respect to the conversion of acetate to cholesterol by the fetus and the mother. The fetus in both species forms its own cholesterol as well as incorporates the maternal cholesterol into fetal tissues. The placenta in either species appears to be unable to form cholesterol from acetate to any appreciable extent. In the human fetus very small conversions of acetate and cholesterol to neutral steroid hormones have been demonstrated by the use of perfusion techniques (4–6) but similar neutral steroids were not obtained in the liver or adrenal of the fetus with the *in vivo* method used in the present study.

Acknowledgments

The authors wish to thank Dr. Wendell Neimann of the Laboratory for Experimental Medicine and Surgery in Primates (Sterling Forest, New York) for the valuable assistance rendered in the course of these studies.

References

1. Merkatz, I. R., and C. G. Beling, *J Reprod Fertil (Suppl)* **6:** 129, 1969.
2. Venning, E. H., *J Biol Chem* **126:** 595, 1938.
3. Merkatz, I. R., K. Leung, F. Fuchs, and S. Solomon, *In* Goldsmith, E. I., and J. Moor-Jankowski (eds.), Medical Primatology, 1970, Proceedings 2nd Conference on Experimental Medicine and Surgery in Primates, S. Karger, Basel, 1971, p. 571.
4. Solomon, S., C. E. Bird, W. Ling, M. Iwamiya, and P. C. M. Young, *Recent Progr Hormone Res* **23:** 297, 1967.
5. Archer, D. F., R. S. Mathur, N. Wiqvist, and E. Diczfalusy, *Acta Endocrinol (Kbh)* **66:** 666, 1971.
6. Telegdy, G., J. W. Weeks, D. F. Archer, N. Wiqvist, and E. Diczfalusy, *Acta Endocrinol (Kbh)* **63:** 119, 1970.
7. Bloch, K., *J Biol Chem* **157:** 661, 1945.
8. Hellig, H., D. Gattereau, Y. Lefebvre, and E. Bolté, *J Clin Endocrinol Metab* **30:** 624, 1970.
9. Plotz, E. J., J. J. Kabara, M. E. Davis, G. V. LeRoy, and K. G. Gould, *Am J Obstet Gynec* **101:** 534, 1968.
10. Davis, E. M., E. J. Plotz, G. V. LeRoy, R. G. Gould, and H. Werbin, *Am J Obstet Gynec* **72:** 740, 1956.
11. Stern, M. D., M. L. Givner, and S. Solomon, *Endocrinology* **83:** 348, 1968.
12. Ruse, J. L., and S. Solomon, *Biochemistry* **5:** 1065, 1966.
13. Carroll, K. K., *J Lipid Res* **2:** 155, 1961.
14. Engel, L. L., C. B. Cameron, A. Stoffyn, J. A. Alexander, O. Klein, and N. D. Trofimow, *Anal Biochem* **2:** 114, 1961.
15. Kabara, J. J., and J. T. McLaughlin, *J Lipid Res* **2:** 283, 1961.
16. Shapiro, L., J. A. Jastremsky, D. A. Eggen, and D. Kritchevsky, *Lipids* **3:** 2, 136, 1967.
17. Villee, C. A., and S. C. Tsai, *In* Pecile, A., and C. Finzi (eds.), The Feto-Placental Unit, Excerpta Medica Foundation, 1969, p. 110.
18. Kabara, J. J., and N. T. Werthessen, *Lipids* **2:** 6, 4[illegible] 1967.

VARIATION IN HYDROGEN ION CONCENTRATION AND TOTAL ACIDITY IN VINEGAR

TREVA C. KINTNER AND MARGARET MANGEL
Department of Home Economics, University of Missouri, Columbia, Missouri

(Manuscript received June 17, 1952)
Food Research 17: 456-9, (1952)

In connection with research on cooked salad dressing, variation in the pH of the final product was shown to be related to the pH of the vinegar used. Considerable variation in pH was found in the same type of vinegar and even in that bottled under the same brand name. Most reports on vinegar in current literature are based on research completed as long as 50 years ago. More data are available on total acidity, which is controlled by law, than on pH. Figures for the latter are usually given as averages with no information on variation among types and brands of vinegars. The present study was made to obtain a range of values for pH and percent acetic acid for types of vinegar on the current retail market. Of these, cider or apple vinegar is the most widely used. Vinegars have also been made from dilute ethyl alcohol, malt infusion, sugar, molasses, grapes, oranges, bananas, honey, dates, raisins, pears, apricots, peaches, and pineapple. The vinegar which is cheapest and most often used by commercial industries is the distilled or spirit vinegar which usually contains a high percent of acetic acid. A distilled sugar or acid vinegar, with caramel flavoring added, comes in open barrels and is available in states which have no laws opposing the sale of compounded vinegars. Other flavorings as garlic, eschalot, mint, basil, tarragon, dill, thyme, marjoram, or herb salad are found in wine vinegars while tarragon is often added to malt vinegars. The definitions of vinegar, its uses, methods of preparation and analyses have been reviewed by Brannt *(3)*, Jacobs *(4)*, Joslyn *(5)*, Triebold *(6)*, Wetherill *(8)*, and Woodman *(10)*.

EXPERIMENTAL PROCEDURE

The hydrogen ion concentration of the vinegar was measured electrometrically by the glass electrode at 25°C. on the Beckman pH meter. The total acidity was measured as recommended by the Association of Official Agricultural Chemists *(2)* and computed and reported as percent by weight of acetic acid. The acid neutralized during production of the vinegar was estimated as acetate by substitution of the experimentally determined pH and total acidity into the Henderson Hasselbalch equation.

RESULTS AND DISCUSSION

Table 1 shows the hydrogen ion concentration and the total titratable acidity of all of the vinegars studied. The mean pH of all of the vinegars was 3.05, with a range of 2.55 to 3.50. This mean is comparable to average pH's, 3.0 and 2.9, reported by West *(7)* and the American Home Economics Association *(1)*. Most of the bottled cider vinegars, with a mean pH of 3.25, and a range of 3.07-3.50 had a lower hydrogen ion concentration than other kinds tested. The variation in pH of open barrel cider vinegar was greater than for bottled, as shown by a pH range of 2.85 to 3.30 for 8

samples, and the mean pH, 3.08, was lower. The mean pH of white wine vinegar was 3.00, while that of the red wine was more acid at 2.87. These vinegars were quite uniform in hydrogen ion concentration. Malt, peach, pear, and apricot vinegars had pH's similar to bottled cider vinegar. The distilled vinegars were the most acid of the vinegars tested, with a mean pH of 2.74 and a range for 8 samples of 2.55 to 2.90. The distilled sugar had a higher mean pH at 2.80 than the regular distilled at 2.64.

TABLE 1

pH and percent acetic acid of vinegars

Type of vinegar	Description	pH: Number of samples	pH: Mean	pH: Range	Acetic acid: Number of samples	Acetic acid: Mean	Acetic acid: Range
						%	%
Cider	Bottled	18	3.25	3.07-3.50	9	4.4	3.9-5.0
	Open barrel	8	3.08	2.85-3.30	5	4.4	3.8-5.0
	All cider	26	3.19	2.85-3.50	14	4.4	3.8-5.0
Wine	Red bottled	6	2.87	2.80-2.97	6	5.1	4.7-5.5
	White bottled	3	3.00	2.96-3.04	3	4.9	4.7-5.0
	All wine	9	2.91	2.80-3.04	3	5.0	4.7-5.5
Malt	Bottled	3	3.07	3.02-3.10	3	5.3	5.2-5.6
Peach	Bottled	1	2.98	.....	1	5.4	.....
Pear	Bottled	1	3.25	.....	1	4.4	.....
Apricot	Bottled	1	3.10	.....	1	5.0	.....
Distilled	Regular bottled	5	2.64	2.55-2.80	4	5.1	5.0-5.2
	Sugar open barrel	3	2.80	2.65-2.90	2	3.6	3.5-3.7
	All distilled	8	2.74	2.55-2.90	6	4.6	3.5-5.2
All vinegars		49	3.05	2.55-3.50	35	4.7	3.5-5.6

A minimum of 4 g. of acetic acid in 100 ml., or an acetic acid content of 4%, is required by law. The mean per cent acetic acid for 35 samples tested was 4.7, with a range of from 3.5 to 5.6. These values exclude two samples of wine vinegars, with 6.4% acetic acid, which had been stored for several years. A mean percent acetic acid for all cider vinegars tested was 4.4, with a range of 3.8 to 5.0. Winton and Winton *(9)* reported the percent acetic acid for cider vinegars at 4.84. There was little difference in the mean total acidity of bottled and open barrel cider vinegars. The red wine was similar in total acidity to the white wine vinegars with a mean of 5.0 and a range of 4.7 to 5.5%. This mean is considerably lower than that of 5.57 reported by Winton and Winton. The samples of malt vinegar averaged 5.3% acetic acid in contrast to the 4.23 reported by the above mentioned authors. The peach, apricot, and pear vinegars showed total acidities of 5.4, 5.0, and 4.4%, respectively. The distilled vinegars tested showed a range in percent acetic acid from 3.5 to 5.2, with a mean of 4.6. The total acidity of the bottled distilled vinegars was between

5.0 and 5.2%, but that for distilled sugar vinegar, 3.5 to 3.7, was lower than the legal limit. The difference between the mean total acidity, 5.1%, of bottled distilled vinegar and that of 6.34% reported by Winton and Winton, is probably due to an attempt by the manufacturer to produce a uniform 5% bottled distilled vinegar.

A preliminary examination of the relationship between total acidity and pH was made, using scatter diagrams. When the mean values of the different types of vinegar were compared, the relationship was similar for all types except for the sugar distilled vinegar. When the values for the individual samples were plotted, the pH of all distilled vinegars was lower in relation to total acidity than those of fruit and malt vinegars. Open barrel vinegars, both the sugar distilled and cider, were more variable and showed less consistent relationship between pH and total acidity than the other vinegars.

An examination of the relationship between total acidity and pH by substitution of these values into the Henderson-Hasselbalch equation indicated a greater neutralization of acid in those vinegars made from fruits than in distilled type vinegars. The estimated acetate in terms of millimolar concentration is shown in Table 2. The mean acetate concentration

TABLE 2

Concentration of acetate in vinegars as estimated by substitution into Henderson-Hasselbalch equation

Type of vinegar	Description	Number of samples	Acetate in millimoles	
			Mean	Range
Cider	Bottled	6	19.6	15.3-24.9
	Open barrel	5	16.2	8.6-28.9
	All cider	11	18.1	8.6-28.9
Wine	Red	6	10.9	9.2-12.7
	White	3	14.5	13.0-15.9
	All wine	9	12.1	9.2-15.9
Malt	Bottled	3	18.2	17.0-19.0
Peach	Bottled	1	14.9	.....
Pear	Bottled	1	22.5	.....
Apricot	Bottled	1	18.2	.....
Distilled	Regular bottled	4	5.9	5.2-7.2
	Sugar open barrel	2	6.4	4.8-8.0
	All distilled	6	6.1	4.8-8.0
All vinegars		32	14.2	4.8-28.9

for all vinegars was 14.2, with a range of from 4.8 to 28.9 millimoles. The acetate estimate for cider, wine, and other fruit and malt vinegars ranged from 8.6 to 28.9 millimoles, while the distilled was considerably lower with a range of from 4.8 to 8.0 and a mean of 6.1 millimoles.

SUMMARY

A survey of the literature indicated a need for more information on pH and total acidity of the many types of vinegar available at the present time. The mean pH of 49 samples of 7 different types of vinegars was 3.05. The pH values for the individual samples ranged from 2.55 to 3.50. The percent of acetic acid for 35 of these samples ranged from 3.5 to 5.6 with a mean of 4.7%. The low pH's obtained in relation to total acidity for distilled vinegars might be explained by the low salt formation in vinegar of this type.

The range in both pH and total acidity obtained in this study is smaller than that reported previously, which may indicate a trend toward uniformity in vinegars on the market today.

LITERATURE CITED

1. American Home Economics Association. *Handbook of Food Preparation.* Revised 1950, A.H.E.A., Washington, D. C.
2. Association of Official Agricultural Chemists. *Official Methods of Analysis.* 7th Ed., 1950, A.O.A.C., Washington, D. C.
3. Beannt, W. R. *A Practical Treatise on the Manufacture of Vinegar.* 3rd Ed., 1914, Henry Carey Baird and Co., Philadelphia, Penn.
4. Jacobs, M. B. *The Chemistry and Technology of Food and Food Products.* 2nd Ed., Volume 2, 1951, Interscience Publishers Inc., New York.
5. Joslyn, M. A. *Methods in Food Analysis.* 1950, Academic Press, Inc., New York.
6. Treibold, H. O. *Quantitative Analysis.* 2nd Printing, 1947, D. Van Nostrand Co., Inc., New York.
7. West, E. S. *Physical Chemistry.* 1942. The Macmillan Co., New York.
8. Wetherill, C. M. *The Manufacture of Vinegar.* 1860, Lindsay and Blakiston, Philadelphia.
9. Winton, A. L., and Winton, K. B. *The Analysis of Foods.* 1945, John Wiley and Sons, Inc., New York.
10. Woodman, A. G. *Food Analysis.* 4th Ed., 1941, McGraw-Hill Book Co., New York.

Russian Translation

Effect of Vitamin C on the Hypercholesterolemia of Fasting Rabbits

V.N. Kolmakov (From the Chair of Biological Chemistry of the Leningrad Sanitary-Hygienic Medical Institute)

Source: Voprosy Meditsinskoy Khimii Vol. 3, No. 6, pp. 414-19, 1957.

Disruption of cholesterol metabolism lies at the basis of the development of atherosclerosis. For this reason, a study of the possible ways of affecting the disrupted cholesterol metabolism is of great interest, and particularly the study of vitamins, which represent natural factors for the regulation (control) of metabolism.

There are indications in the literature that vitamin C reduces hypercholesterolemia (1-4).

In view of the fact that most authors have limited their investigations to the study of the effect of vitamin C on nutritional hypercholesterolemia, we have studied the effect of vitamin C on the development of hypercholesterolemia in fasting rabbits and on the metabolism of cholesterol in the liver of these animals.

Methods and Results of Studies

Effect of vitamin C on the development of hypercholesterolemia in fasting rabbits. Hypercholesterolemia in fasting rabbits is a convenient experimental model for studying disturbances of cholesterol metabolism, since it develops very rapidly and is accompanied by a sharp increase in the content of cholesterol in blood.

S.V. Nedzvetskii (5) was the first one to show that hypercholester-

olemia in fasting rabbits is the result (consequence) of a developing hyperketonemia. Hence, it was of interest to study the effect of vitamin C on the development of these metabolic disturbances.

Significant individual variations in the content of blood cholesterol are observed in fasting rabbits. Therefore, tests were carried out as follows: blood was taken from the heart of the rabbit, and it was then left without food (receiving only water) for 5 days. On the 3rd and 5th day of fasting, blood was taken from the heart. The cholesterol content was determined in the plasma of the collected blood by previously described methods (5), and the content of ketone bodies was determined in whole blood. The rabbit was then kept on the usual diet for 2 weeks, after which a similar test was carried out, in which a daily dose of 200 mg vitamin C was injected subcutaneously. In a similar manner, the development of hypercholesterolemia and hyperketonemia was studied in some rabbits during fasting and introduction of vitamin C, and after the same period of time without introduction of the vitamin.

The results of several typical tests are shown in Table 1. Analogous results were obtained in 14 out of 18 tests. In 2 tests, vitamin C did not affect the development of hypercholesterolemia and in 2 tests it even enhanced the latter. In none of the tests was hyperketonemia influenced by vitamin C. The data given here show that, in most cases, vitamin C reduces hypercholesterolemia in fasting rabbits.

Table 1

Effect of Vitamin C on The Development of Hypercholesterolemia and Hyperketonemia in Fasting Rabbits

1 № опыта	2 Время взятия крови	3 Голодание без дачи витамина С		4 Голодание с дачей витамина С	
		5 кетоновые тела (в мг% ацетона)	6 холестерин (в мг%)	5 кетоновые тела (в мг% ацетона)	6 холестерин (в мг%)
1	7 До голодания	0,37	35	0,37	24
	8 Через 3 дня от начала голодания	0,85	54	0,63	35
	9 Через 5 дней от начала голодания	1,13	50	1,46	33
2	7 До голодания	0,30	20	0,34	34
	8 Через 3 дня от начала голодания	0,51	67	1,03	52
	9 Через 5 дней от начала голодания	1,14	95	1,03	63
3	7 До голодания	0,13	32	0,22	29
	8 Через 3 дня от начала голодания	1,28	141	0,51	63
	9 Через 5 дней от начала голодания	0,93	171	1,14	89

1 -- Test no.
2 -- Time of blood collection
3 -- Fasting without vitamin C addition
4 -- Fasting with vitamin C addition
5 -- Ketone bodies (in mg % acetone)
6 -- Cholesterol (in mg %)
7 -- Before fasting
8 -- 3 days after start of fasting
9 -- 5 days after start of fasting

Mechanism of Action of Vitamin C

Effect of vitamin C on cholesterol excretion in bile. A.L. Myasnikov (6) has shown that vitamin C improves the function of the liver and increases the excretion of cholesterol in bile, which results in a reduction of hypercholesterolemia.

For a more detailed experimental study of this problem, the following tests were set up: on the 5th day of fasting, with simultaneous daily

subcutaneous injection of 200 mg vitamin C, the rabbit was killed by introduction of air into the vein. The liver was quickly removed, the gall bladder was opened and bile was collected in a test tube. In the bile thus obtained the cholesterol content was determined by the method of Sperry and Brand (7).

The cholesterol content was also studied in the gall bladder bile of rabbits killed on the 5th day of fasting (which did not receive vitamin C) and of rabbits kept on a usual diet. The data obtained are shown in Table 2.

Table II

Effect of Vitamin C on The Cholesterol Content of Gall Bladder Bile of Fasting Rabbits

1 Условия опыта	2 Чис. о опытов	3 Содержание холестерина в желчи (в мг%)
Обычное питание . . . 4	15	68,8±25,3
Голодание 5 дней . . . 5	12	73,5±15,8
Голодание 5 дней с введением витамина С . . . 6	20	81,0±24,7

1 -- Test conditions
2 -- Number of tests
3 -- Cholesterol content in bile (in mg %)
4 -- Usual diet
5 -- Fasting for 5 days
6 -- Fasting for 5 days with introduction of vitamin C

Although the cholesterol content in bladder bile varies significantly in different rabbits, the data obtained show that vitamin C does not exert a significant effect on the cholesterol content of bladder bile of

fasting rabbits, and consequently also on the excretion of cholesterol with bile.

Effect of vitamin C on the cholesterol content in the liver of fasting rabbits. We have also studied the effect of vitamin C on the content of cholesterol in the liver of fasting rabbits.

Tests were carried out in the same way as in the study of the cholesterol content of bile. The cholesterol content in the liver of rabbits was determined as follows: a weighed sample (2-5 g) of liver tissue was hydrolyzed with 15 ml of a 15% aqueous NaOH solution for 6 hours on a boiling water bath. The hydrolyzate was acidified by adding 20 ml of a 25% H_2SO_4 solution and extracted 3 times with ether. Cholesterol was determined colorimetrically in the ether extract by the Libermann-Burchard reaction in a Col-1 M colorimeter with a No. 2 light filter (λ_{eff} = 633 mμ).

The data obtained are shown in Table 3.

Table 3

Effect of Vitamin C on The Content of Cholesterol in The Liver of Fasting Rabbits

1 Условия опыта	2 Число опытов	3 Содержание холестерина в печени (в мг%)
4 Обычное питание	25	232 ±38,2
5 Голодание 5 дней	24	332,2±94,2
6 Голодание 5 дней с введением витамина С	22	385,5±86,8

1 -- Test conditions
2 -- Number of tests
3 -- Cholesterol content in liver (in mg 5)
4 -- Usual diet
5 -- Fasting for 5 days
6 -- Fasting for 5 days with introduction of vitamin C

From the data given in the table we can see that fasting results in an increased cholesterol content in the liver of rabbits, as pointed out by many other authors. Supply of vitamin C to fasting rabbits leads to an even greater increase of the cholesterol content in the liver.

Effect of Vitamin C on Cholesterol Metabolism in the Liver Tissue of Fasting Rabbits

There are indications that the liver is the main source of endogenous plasma cholesterol (8). For this reason, we can assume that vitamin C lowers hypercholesterolemia by influencing the metabolism of cholesterol in the liver.

Tests for studying cholesterol metabolism in liver tissue were carried out as follows: the rabbit was killed by introducing air into the vein, the liver was quickly removed and cut up with scissors into small pieces in the cold. From the fine paste (brei) thus obtained, 3 parallel samples of about 5 g each were taken. To the first sample caustic alkali was immediately added and the original cholesterol content was determined. To the second sample, 5 ml of a 5% Na acetate solution was added, and the synthesis of cholesterol was studied in this sample. To the third sample, 5 ml of a 1% KH_2PO_4 solution was added, and the breakdown of cholesterol was studied in this sample. All samples were incubated in a O_2 atmosphere at 37-40°C for 3 hours. At the end of incubation 15 ml of a 15% NaOH solution was added to all samples, and the cholesterol content was determined by the method described above. The cholesterol content was calculated per 100 g of tissue weight.

To study the effect of vitamin C on cholesterol metabolism in the liver of rabbits. Rabbits in the first group were kept on a usual diet. A

total of 18 tests were carried out, and data from several typical tests are shown in Table 4.

Table 4

Cholesterol Content in The Liver Brei of Rabbits Kept on A Usual Diet Under Different Incubation Conditions

2 № опыта	1 Содержание холестерина (в мг%)		
	3 исходное	4 инкубация с ацетатом (синтез)	5 инкубация с фосфатом (распад)
1	193,0	233,0	104,0
2	195,2	209,4	134,5
3	220,0	276,0	124,0
4	279,0	275,6	221,1
5	290,1	225,6	214—252

1 -- Cholesterol content (in mg 5)
2 -- Test no.
3 -- Original content
4 -- Incubation with acetate (synthesis)
5 -- Incubation with phosphate (breakdown)

From the data obtained we can see that, during incubation of rabbit liver tissue with acetate, an increase in the cholesterol content takes place, i.e. cholesterol is synthesized from the added acetate. When liver tissue is incubated with phosphate, the cholesterol content decreases, i.e. a breakdown of cholesterol present in liver tissue takes place.

Gold et al. (9) found that when rabbits are fed large amounts of cholesterol, the content of the latter in the blood and liver increases. Simultaneously, the synthesis of cholesterol from C^{14}-acetate in the liver of these animals is inhibited. We have also been able to note a relationship between the cholesterol content of liver tissue and the ability of this tissue to synthesize cholesterol from the added acetate. Thus, when the content of cholesterol in the liver increased (Table 4, test 4),

its synthesis from added acetate was not observed. When the cholesterol content of the liver was even higher (Table 4, test 5), addition of acetate was even accompanied by a breakdown of the cholesterol present in the liver. The second group included fasting rabbits (killed on the 5th day of fasting).

A total of 12 tests were carried out. Data from several typical tests are shown in Table 5.

Table 5

Cholesterol Content in Liver Brei of Fasting Rabbits
Under Different Incubation Conditions

2 № опыта	1 Содержание холестерина (в мг%)		
	3 исходное	4 инкубация с ацетатом (синтез)	5 инкубация с фосфатом (распад)
1	226,6	261,4	226,6
2	236,8	273,2	236,8
3	2[illegible]8,4	258,3	261,0
4	348,3	313,2	341,7
5	694,5	244--326	673,4

1 -- Cholesterol content (in mg 5)
2 -- Test no.
3 -- Original content
4 -- Incubation with acetate (synthesis)
5 -- Incubation with phosphate (breakdown)

When we examine the data obtained, we notice that the breakdown of cholesterol in the liver of fasting rabbits is inhibited, since the cholesterol content is not reduced during incubation of the liver brei with phosphate. However, it should be noted that in 3 cases out of 12 the breakdown of cholesterol in the liver of fasting rabbits was not disturbed. An inhibition of cholesterol breakdown in the liver of fasting rabbits was also observed by Wagner (10). Van Bruggen et al (11) noted an inhibition

of cholesterol breakdown in the organism of intact fasting rats.

There are indications in the literature that the liver is one of the important organs taking part in the removal of excess cholesterol from blood plasma (12). For this reason, the disruption of cholesterol breakdown in the liver of fasting rabbits is probably one of the causes of the hypercholesterolemia which develops in this case.

The data obtained in our tests also indicate that cholesterol synthesis in the liver of fasting rabbits depends upon the original content of cholesterol in the liver. In case of a low cholesterol content (Table 5, tests 1 and 2), its synthesis takes place from the added acetate. An increase in cholesterol content (Table 5, tests 3 and 4) results in an inhibition of its synthesis from added acetate. Finally, in case of a high cholesterol content (Table 5, test 5), the addition of acetate is accompanied by a breakdown of cholesterol.

The third group included fasting rabbits receiving simultaneously a daily dose of 200 mg vitamin C by subcutaneous injection, and which were killed on the 5th day of fasting. A total of 12 tests were carried out. Data from several typical tests are shown in Table 6.

From the data reported here we can see that vitamin C restores the breakdown of cholesterol in the liver of fasting rabbits, since the cholesterol content in liver tissue is reduced during incubation with phosphate. However, it should be noted that a restoration of cholesterol breakdown was not observed in 2 out of 12 cases. Synthesis of cholesterol from added acetate was not observed in any of these tests; this probably depends on the high original content of cholesterol in the liver. It was noted that, in case of a high original cholesterol content (Table 6, tests 2-5),

Table 6

Cholesterol Content in The Liver Brei of Fasting Rabbits
With Simultaneous Injection of Vitamin C Under
Different incubation conditions

1 № опыта	2 Содержание холестерина (в мг%)		
	3 исходное	4 инкубация с ацетатом (синтез)	5 инкубация с фосфатом (распад)
1	258,0	258,0	228,7
2	270,5	255,5	213,9
3	410,3	378,8	325,0
4	579,0	392,6	411,8
5	625,0	571—615	369—470

1 -- Cholesterol content (in mg 5)
2 -- Test no.
3 -- Original content
4 -- Incubation with acetate (synthesis)
5 -- Incubation with phosphate (breakdown)

addition of acetate is accompanied by a breakdown of cholesterol present in liver tissue.

Discussion of Results

As can be seen from the data reported here, injection of vitamin C inhibits the development of hypercholesterolemia in fasting rabbits. This effects of vitamin C can be explained as follows. The cholesterol content in the bladder bile of fasting rabbits receiving simultaneously vitamin C does not differ significantly from the cholesterol content in the bladder bile of fasting rabbits which do not receive the vitamin. For this reason, the reduction of hypercholesterolemia under these test conditions can hardly be explained as due to a greater excretion of cholesterol with bile. The increased cholesterol content in the liver of rabbits, which daily received vitamin C during fasting, as compared to the cholesterol content in the liver of rabbits which did not get the vitamin, gives us reason to believe that vitamin C contributes to the retention by the liver of excess cholesterol from blood. It is possible that this factor is responable for the reduction of hypercholesterolemia in fasting rabbits receiving simultaneously vitamin C. Under these conditions, the liver of rabbits loses its ability to synthesize cholesterol from added acetate, which, possibly, is due to the higher cholesterol content in the liver. At the same time, vitamin C exerts an effect on the opposite process; it restores the breakdown of cholesterol in liver tissue, which is inhibited in fasting rabbits. Consequently, vitamin C must be considered as an important factor in the regulation of cholesterol metabolism.

Conclusions

In studying the effect of vitamin C on the development of hypercholesterolemia in fasting rabbits, it was found that vitamin C:

1. Inhibits the development of hypercholesterolemia in fasting rabbits.

2. Does not exert any significant effect on the excretion of cholesterol with bile.

3. Contributes to the retention by the liver of excess cholesterol from blood.

4. Restores the breakdown of cholesterol in the liver, inhibited in fasting rabbits.

Bibliography

1. Myasnikova I. A. Trudy Voenno-morsk. med. akad. 1947, vol. 8, p. 140.
2. Loybman G. I., Berkovskii E. m. Farmakol. i toksikol. 1945, vol. 14, no. 2, p. 17.
3. Myasnikova I. A. Trudy Akad. med. nauk SSSR, Moscow, 1952, vol. 20, no. 2, p. 103.
4. Tyapina L. A. Ibid. p. 108.
5. Nedzvetskii S. V. Arkh. patol. 1950, vol. 12, no. 5, p. 34.
6. Myasnikov A.L. Klin. med. 1951, vol. 32, no. 6, p. 9.
7. Sperry W. R., Brand F. J. Biol. Chem., 1943, vol. 150, p. 315.
8. Eckles M. E., Taylor C. B., Campbell, D. J., Gould R. G. J. Lab. Clin. Med., 1955, vol. 46, p. 359
9. Gould R. G., Taylor C. B., Hagerman J. S., et al. J. Biol. Chem., 1953, vol. 201, p. 519.
10. Wagner O. Citation from Chem. Abstr. 1930, vol. 24, p. 2746.
11. Van Bruggen J. T., Hutchens T. T., Claycomb C. K. et al. J. Biol. Chem., 1952, vol. 196, p. 389.
12. Hotta S., Chaikoff T. L. Arch. Biochem. Biophys., 1955, vol. 56, p. 28.

1957

ВЛИЯНИЕ ВИТАМИНА С НА ГИПЕРХОЛЕСТЕРИНЕМИЮ У ГОЛОДАЮЩИХ КРОЛИКОВ

В. Н. Колмаков

Кафедра биологической химии Ленинградского санитарно-гигиенического медицинского института

Voprosy Med. Khim. 3, 414-19 (1957)

Нарушение холестеринового обмена лежит в основе развития атеросклероза. Поэтому большой интерес представляет изучение путей воздействия на нарушенный холестериновый обмен, особенно изучение влияния витаминов, являющихся естественными факторами регуляции обмена веществ.

В литературе имеются указания, что витамин С снижает гиперхолестеринемию [1—4].

Поскольку большинство авторов ограничило свои исследования изучением влияния витамина С на пищевую гиперхолестеринемию, нами проведены исследования влияния витамина С на развитие гиперхолестеринемии у голодающих кроликов и на обмен холестерина в печени этих животных.

Методы и результаты исследований

Влияние витамина С на развитие гиперхолестеринемии у голодающих кроликов. Гиперхолестеринемия у голодающих кроликов является удобной экспериментальной моделью для изучения нарушений холестеринового обмена, так как она развивается очень быстро и сопровождается резким повышением содержания холестерина в крови.

С. В. Нельзевский [5] впервые показал, что гиперхолестеринемия у голодающих кроликов является следствием развивающейся гиперкетонемии. Отсюда представляло интерес изучить влияние витамина С на развитие этих нарушений обмена.

У голодающих кроликов имелись значительные индивидуальные колебания содержания холестерина в крови. Поэтому опыты ставились следующим образом: у кролика брался кровь из сердца, а затем он оставлялся без пищи (получал только воду) в течение [illegible] дней, а [illegible] дней брался кровь из сердца. В плазме крови определялось содержание холестерина. [illegible] Ранее [illegible] определялось содержание холестерина [illegible] кролик в течение 2 недель содержался на [illegible] без витамина С. [illegible] ставился аналогичный опыт, но только с введением [illegible] витамина С подкожно по 200 мг. У некоторых кроликов подобный опыт [illegible] сначала получалось [illegible] гиперхолестеринемии и гиперкетонемии при [illegible] витамина С, а через такой же промежуток времени [illegible] витамина.

В табл. 1 приведены результаты [illegible] типичных опытов. Аналогичные результаты получены в 14 опытах из 18. В 2 опытах витамин С [illegible] гиперхолестеринемию, а в 2 опытах даже [illegible] гиперхолестеринемию. Но в одном опыте не было отмечено влияния витамина С на гиперкетонемию.

Приведенные данные доказывают, что в большинстве случаев витамин С снижает гиперхолестеринемию у голодающих кроликов.

Table 1

Таблица 1

Влияние витамина С на развитие гиперхолестеринемии и гиперкетонемии у голодающих кроликов

№ опыта	Время взятия крови	Голодание без дачи витамина С		Голодание с дачей витамина С	
		кетоновые тела (в мг% ацетона)	холестерин (в мг%)	кетоновые тела (в мг% ацетона)	холестерин (в мг%)
1	До голодания	0,37	35	0,37	24
	Через 3 дня от начала голодания	0,85	54	0,63	35
	Через 5 дней от начала голодания	1,13	50	1,46	33
2	До голодания	0,30	20	0,34	34
	Через 3 дня от начала голодания	0,51	67	1,03	52
	Через 5 дней от начала голодания	1,14	95	1,03	63
3	До голодания	0,13	32	0,22	29
	Через 3 дня от начала голодания	1,28	141	0,51	63
	Через 5 дней от начала голодания	0,93	171	1,14	89

О механизме действия витамина С

Влияние витамина С на выделение холестерина с желчью. А. Л. Мясников [6] указывает, что витамин С улучшает функцию печени и усиливает выделение холестерина с желчью, что и ведет к снижению гиперхолестеринемии.

Table 2

Таблица 2

Влияние витамина С на содержание холестерина в пузырной желчи голодающих кроликов

Условия опыта	Число опытов	Содержание холестерина в желчи (в мг%)
Обычное питание	15	68,8 ± 25,3
Голодание 5 дней	12	73,5 ± 15,8
Голодание 5 дней с введением витамина С	20	81,0 ± 24,7

Для более детального экспериментального изучения этого вопроса были поставлены следующие опыты: на 5-й день голодания с одновременным введением витамина С (ежедневно по 200 мг подкожно) кролик забивался введением воздуха в вену. Печень быстро извлекалась, вскрывался желчный пузырь и желчь собиралась в пробирку. В полученной желчи определялось содержание холестерина по методу [illegible] [7].

Было также [illegible] содержание холестерина [illegible] кроликов, забитых на 5-й день голодания [illegible] и кроликов, находившихся на обычном питании.

Полученные данные приведены в табл. 2.

Хотя содержание [illegible] лется у разных [illegible] не оказывает существенного влияния на [illegible] зырной желчи голодающих кроликов, и [illegible] холестерина с желчью.

Влияние витамина С на содержание холестерина [illegible] щих кроликов. Нами было изучено также [illegible] жание холестерина в печени у голодающих [illegible]

[illegible] как при изучении содержания холестерина в желчи. Определение содержания холестерина в печени кроликов проводилось следующим образом: навеска печеночной ткани 2—5 г гидролизовалась с 15 мл 15% водного раствора NaOH в течение 6 часов на кипящей водяной бане. Гидролизат подкислялся добавлением 20 мл 25% раствора H_2SO_4 и трижды экстрагировался эфиром. В эфирном экстракте определялся холестерин с помощью реакции Либермана-Бурхарда колориметрированием в колориметре Кол-1М со светофильтром № 2 ($\lambda_{эф}$ 632 нм).

Полученные данные приведены в табл. 3.

Таблица 3 Table 3

Влияние витамина С на содержание холестерина в печени голодающих кроликов

(1) Условия опыта	(2) Число опытов	(3) Содержание холестерина в печени (в мг%)
(4) Обычное питание	25	232 ± 38,2
(5) Голодание 5 дней	24	332,2 ± 94,2
(6) Голодание 5 дней с введением витамина С	22	385,5 ± 86,8

Из приведенных данных видно, что голодание ведет к повышению содержания холестерина в печени кроликов, на что указывают и многие другие авторы. Дача голодающим кроликам витамина С ведет к еще большему повышению содержания холестерина в печени.

Влияние витамина С на обмен холестерина в ткани печени голодающих кроликов

Имеются указания, что печень является основным источником эндогенного холестерина плазмы [8]. Поэтому можно думать, что витамин С снижает гиперхолестеринемию, влияя на обмен холестерина в печени.

Опыты по изучению обмена холестерина в ткани печени были поставлены следующим образом: кролик забивался введением воздуха в вену. Печень быстро извлекалась и измельчалась ножницами на холоду. Из полученной кашицы отбирались 3 параллельные пробы около 5 г каждая. Первая проба сразу же заливалась щелочью и в ней определялось исходное содержание холестерина. Ко второй пробе добавлялось 5 мл 5% раствора CH_3COONa и в ней изучался синтез холестерина. К третьей пробе добавлялось 5 мл 1% раствора KH_2PO_4 и в ней изучался распад холестерина. Все пробы инкубировались в атмосфере O_2 при температуре 37—40° в течение 3 часов. По окончании инкубации все пробы заливались 15 мл 15% раствора NaOH и определялось содержание холестерина описанным выше способом. Содержание холестерина рассчитывалось на 100 г веса ткани.

Таблица 4 Table 4

Содержание холестерина в кашице печени кроликов, находящихся на обычном питании при различных условиях инкубации

(2) № опыта	(1) Содержание холестерина (в мг%)		
	(3) исходное	(4) инкубация с ацетатом (синтез)	(5) инкубация с фосфатом (распад)
1	193,0	233,0	164,0
2	195,2	209,4	134,5
3	225,0	276,0	124,0
4	279,0	275,6	221,1
5	290,1	225,6	214—252

Для изучения влияния витамина С на обмен холестерина в печени голодающих кроликов были поставлены опыты на 3 группах кроликов.

В первой группе кролики находились на обычном питании. Всего поставлено 18 опытов. Данные нескольких типичных опытов приведены в табл. 4.

Из полученных данных видно, что при инкубации ткани печени кроликов с ацетатом происходит нарастание содержания холестерина, т. е. идет синтез последнего из добавленного ацетата. При инкубации ткани печени с фосфатом содержание холестерина снижается, т. е. происходит распад содержащегося в ткани печени холестерина.

Голд с сотрудниками [9] нашли, что при скармливании животным больших количеств холестерина повышается его содержание в крови и в печени. Одновременно происходит угнетение синтеза холестерина из C^{14}-ацетата в печени этих животных. Нам также удалось отметить зависимость между содержанием холестерина в ткани печени и способностью этой ткани к синтезу холестерина из добавленного ацетата. Так, при повышении содержания холестерина в печени (табл. 4, опыт 4) его синтез из добавленного ацетата не наблюдался. При еще большем повышении содержания холестерина в печени (табл. 4, опыт 5) добавление ацетата сопровождалось даже распадом холестерина, содержащегося в печени. Во второй группе находились голодавшие кролики (забиты на 5-й день голодания).

Всего поставлено 12 опытов. Данные нескольких типичных опытов приведены в табл. 5.

Таблица 5

Содержание холестерина в печеночной кашице голодавших кроликов при различных условиях инкубации

№ опыта	Содержание холестерина (мг%)		
	исходное	инкубация с ацетатом (синтез)	инкубация с фосфатом (распад)
1	226,6	261,4	226,6
2	236,8	273,2	236,8
3	2[illegible]8,4	258,3	261,6
4	318,3	343,2	341,7
5	694,5	244–326	673,4

При рассмотрении полученных данных обращает на себя внимание торможение распада холестерина в печени голодающих кроликов, так как не происходит снижения содержания холестерина при инкубации печеночной кашицы с фосфатом. Однако нужно отметить, что в 3 случаях из 12 распад холестерина в печени голодающих кроликов не был нарушен. Угнетение распада холестерина в печени голодающих кроликов наблюдал также Вагнер [10]. Ван Брагтен с сотрудниками [11] отметили торможение распада холестерина в организме интактных голодающих крыс.

В литературе имеются указания, что печень является одним из важнейших органов, участвующих в удалении избытка холестерина из плазмы крови [12]. Поэтому нарушение распада холестерина в печени голодающих кроликов, вероятно, является одной из причин развивающейся при этом гиперхолестеринемии.

Полученные в наших опытах данные указывают также, что синтез холестерина в печени голодающих кроликов зависит от исходного содержания холестерина в печени. При низком содержании холестерина (табл. 5, опыты 1 и 2) происходит его синтез из добавленного ацетата. Повышение содержания холестерина (табл. 5, опыты 3 и 4) ведет к угнетению его синтеза из добавленного ацетата. Наконец, при высоком содержании холестерина (табл. 5, опыт 5) добавление ацетата сопровождается распадом холестерина.

В третьей группе находились кролики, голодавшие и одновременно получавшие витамин С (ежедневно 200 мг подкожно). Забиты на пятый день голодания.

Всего поставлено 12 опытов. Данные нескольких типичных опытов приведены в табл. 6.

Таблица 6 Table 6

Содержание холестерина в печеночной кашице голодавших кроликов с одновременным введением витамина С при различных условиях инкубации

№ опыта	Содержание холестерина (в мг%)		
	исходное	инкубация с ацетатом (синтез)	инкубация с фосфатом (распад)
1	258,0	258,0	228,7
2	270,5	255,5	213,9
3	410,3	378,8	325,0
4	579,0	392,6	411,8
5	625,0	571—615	369—470

Из приведенных данных видно, что витамин С восстанавливает распад холестерина в печени голодающих кроликов, так как при инкубации с фосфатом происходит снижение содержания холестерина в ткани печени. Однако нужно отметить, что в 2 опытах из 12 не наблюдалось восстановления распада холестерина. Синтез холестерина из добавленного ацетата ни в одном опыте не наблюдался. Это, вероятно, зависит от высокого исходного содержания холестерина в печени. Отмечено, что при высоком исходном содержании холестерина (табл. 6, опыты 2—5) добавление ацетата сопровождается распадом содержащегося в ткани печени холестерина.

Обсуждение полученных результатов

Как видно из приведенных данных, введение витамина С задерживает развитие гиперхолестеринемии у голодающих кроликов. Это влияние витамина С можно объяснить следующим образом. Содержание холестерина в пузырной желчи кроликов, голодавших и одновременно получавших витамин С, не отличается существенно от такового в пузырной желчи кроликов, голодавших и не получавших витамина. Поэтому снижение гиперхолестеринемии в этих условиях опыта едва ли можно объяснить усиленным выделением холестерина с желчью. Повышение содержания холестерина в печени кроликов, которые при голодании ежедневно получали витамин С, по сравнению с содержанием холестерина в печени кроликов, не получавших витамина, дает основание думать, что витамин С способствует задержке избытка холестерина из крови печенью. В этом, вероятно, и обусловливается снижение гиперхолестеринемии у голодающих кроликов, одновременно получающих витамин С. В этих условиях печень кроликов утрачивает способность синтезировать холестерин из добавленного ацетата, что, возможно, обусловливается повышенным содержанием холестерина в печени. Одновременно витамин С влияет на противоположный процесс. Он восстанавливает распад холестерина в ткани печени, который угнетен у голодающих кроликов. Следовательно, витамин С следует рассматривать, как важный фактор в регуляции холестеринового обмена.

Выводы

При исследовании влияния витамина С на развитие гиперхолестеринемии у голодающих кроликов установлено, что витамин С:

1) задерживает развитие гиперхолестеринемии у голодающих кроликов;

2) не оказывает существенного влияния на выделение холестерина с желчью;

3) способствует задержке печенью избытка холестерина из крови,
4) восстанавливает распад холестерина в печени, угнетенный у голодающих кроликов.

ЛИТЕРАТУРА Bibliography -

1. Мясникова Н. А. Труды Воен-мор. мед. акад, 1947, т. 8, стр. 11[illegible]. 2. Лойбман Г. И. и Берковский Э. М. Фармакол. и токсикол, 1951, т. 14, № 2, стр. 17.—3. Мясникова Н. А. Труды Акад. мед. наук СССР М., 1952, т. 29, в. 2, стр. 103.—4. Тяпина Л. А. Там же, стр. 108.—5. Недзвецкий С. В. Мед. пат., 1950, т. 12, № 5, стр. 34.—6. Мясников А. Л. Клин. мед., 1951 т. 32, № 6, стр. 9.—7. Sperry W. R., Brand F. Journ. biol. chem., 1943, v. 150, p. 315.— 8. Eckles M. E., Taylor C. B., Campbell D. J., Gould R. G. Journ. lab. clin. med., 1955, v. 46, p. 359. — 9. Gould R. G., Taylor C. B., Hagerman J. S. a oth. Journ. biol. chem., 1953, v. 201, p. 519.—10. Wagner O. (Ber. no Chem. Abstr., 1930, v. 24, p. 2764.—11. Van Bruggen J. T., Hutchens J. T., Claycomb C. K. (a. oth) Journ. biol. chem., 1952, v. 196, p. 389.—12. Hotta S., Chaikoff I. L. Arch. biochem. biophys., 1955, v. 56, p. 28.

Citation from Abstr.

et al.

Поступила 5 XI 1956 г.

THE EFFECT OF VITAMIN C ON THE HYPERCHOLESTEROLEMIA OF FASTING RABBITS

By *V. N. Kolmakov*

Chair of Biochemistry, Leningrad Sanitary Hygienic Medical Institute

Summary

Vitamin C exerts an inhibitory effect on the development of hypercholesterolemia in fasting rabbits. Abatement of the hypercholesterolemia does not occur as a result of an intensified excretion of cholesterol with bile, but is rather connected with a retention by the liver of excess blood cholesterol.

A study was carried out of the cholesterol metabolism in liver tissue of rabbits under different nutritional conditions (normal nutrition, 5 days fasting, 6 days fasting with simultaneous subcutaneous administration of 200.0 mg of vitamin C). It was found that under these conditions vitamin C restores the degradation of cholesterol in liver tissue, which is known to be inhibited in fasting animals.

In experiments on cholesterol synthesis from acetate in liver tissue it was noted that with high concentration of cholesterol in liver the addition of acetate results in degradation of cholesterol.

P. 51040

The influence of dietary fat on fatty acid biosynthesis in the rat

Since the initial report by SCHOENHEIMER AND RITTENBERG[1], many other investigators have observed the interconversion of non-essential fatty acids in the intact rat. The field has recently been reviewed by ELOVSON[2]. The influence of dietary fats upon fatty acid biosynthesis, however, has received relatively little attention. KUISER *et al.*[3] have just reported that fatty acid synthesis from acetate in rats fed triglycerides of C_4–C_8 saturated fatty acids is double that in rats fed C_{10}–C_{16} saturated triglycerides, and 10- to 20-fold higher than in rats fed triolein or trilinolein. Our own observation[4] that hepatic fatty acid synthesis was greater in rats fed medium-chain triglycerides, a synthetic fat composed almost exclusively of triglycerides of C_8 and C_{10} fatty acids, than in rats fed corn or coconut oils, prompted an investigation into the mechanism underlying the reported differences.

Biochim. Biophys. Acta, 116 (1966) 184–185

Four groups of four male Wistar rats (200 gm) were maintained for one week on diets of laboratory chow, or chow augmented with 20% of medium-chain triglycerides, coconut oil or corn oil (Table I). After one week the animals were injected intraperitoneally with sodium [1-^{14}C]acetate (50 μC/100 g) and killed after 4 h. The livers were excised and dissolved in 15% alcoholic KOH. The fatty acids were extracted and subjected to decarboxylation according to the method of PHARES AND LONG[5]. Using apparatus specifically designed for this purpose[6], the fatty acid mixture

TABLE I

FATTY ACID COMPOSITION OF FATS USED IN FEEDING EXPERIMENTS

Fatty acid	*Medium-chain triglyceride (%)*	*Coconut oil (%)*	*Corn oil (%)*
Iodine value	*0.2*	*10*	*126*
6 : 0	Trace	Trace	—
8 : 0	61.3	6.8	—
10 : 0	38.5	7.0	—
12 : 0	Trace	48.5	—
14 : 0	—	17.5	0.2
16 : 0	—	8.7	9.9
16 : 1	—	—	0.5
18 : 0	—	2.0	2.9
18 : 1	—	8.5	30.1
18 : 2	—	1.0	56.2
Other	—	—	—

(2–10 mg) was treated with excess sodium azide (50–150 mg) and 10–15 ml of 15% SO_3 in sulfuric acid. The mixture was heated to 85° and the liberated $^{14}CO_2$ was flushed out with N_2 through a cold finger at −20° (to remove unoxidized material), bubbled through a solution of 5% $KMnO_4$ in 0.5 N H_2SO_4 (to oxidize residual SO_3) and finally trapped in 1 M hyamine hydroxide (*p*-(diisobutyl,cresoxy,ethoxyethyl)-dimethylbenzyl ammonium hydroxide) in methanol. The hyamine–carbonate solution was added to a solution of 0.6% 2,5-diphenyloxazole (PPO) and 0.02% dimethyl 1,4-bis-2(5-phenyloxazolyl) benzene (POPOP) in toluene and assayed by liquid scintillation spectroscopy. The residue from the degradation was neutralized with base, and the recovered liberated amine mixture was oxidized to CO_2 with sulfuric acid and chromate at 150° (ref. 7). The liberated $^{14}CO_2$ was collected in the manner described above and also assayed for radioactivity. One sample of the pooled liver fatty acids from each group was analyzed for its fatty acid content by gas chromatography and the data obtained (Table II) used to calculate the average chain length of the liver fatty acids in each dietary group. The ratio of carboxyl to alkyl specific activities is given in Table III.

It is evident from these results that the ratio of chain elongation (which would give a high carboxyl/chain ratio) to *de novo* synthesis was higher in all three fat-fed groups than it was in the controls, being highest in the corn oil fed group. DAUBEN, HOERGER AND PETERSEN[8] reported that in the fatty acids of mice injected with [1-^{14}C]acetate, the ratio of radioactivity of the carboxyl carbon atom to alkyl carbon-3 was 1 in the isolated palmitic acid and 3 in the isolated stearic acid, indicating specific chain elongation at this stage. Our data suggest that the availability of various specific fatty acids influences the extent and mode of utilization of the administered [1-^{14}C]-

TABLE II

LIVER FATTY ACIDS OF RATS FED VARIOUS FATS

Fatty acid	*Medium-chain triglyceride (%)*	*Coconut oil (%)*	*Corn oil (%)*	*Control (%)*
8 : 0	2.2	1.0	1.3	1.2
10 : 0	3.3	1.6	1.9	2.4
12 : 0	3.7	5.5	2.9	3.5
12 : 1	1.7	4.0	2.4	2.2
14 : 0	2.1	5.1	3.4	3.3
14 : 1	4.4	3.7	—	2.0
16 : 0	23.1	22.6	20.9	22.8
16 : 1	3.2	1.4	1.2	1.5
16 : 2	2.2	1.2	1.1	1.0
18 : 0	28.5	28.7	29.9	31.0
18 : 1	14.8	20.3	29.7	23.5
18 : 2	7.2	-	4.0	3.0
Other	3.3	4.0	1.6	2.2

TABLE III

RATIO OF SPECIFIC ACTIVITIES OF CARBOXYL TO ALKYL CARBONS IN FATTY ACIDS OF RATS FED VARIOUS FATS

Fat	*Ratio carboxyl/alkyl*
Medium-chain triglyceride	4.4 ± 1.3*
Coconut oil	4.6 ± 1.4
Corn oil	9.1 ± 0.9
Control	2.1 ± 0.3

* Standard error.

acetate. Cholesterol biosynthesis from acetate in rats fed corn oil is greater than it is in rats fed coconut oil[4,9,10]. The explanation for this observation may lie in the possibility that in the corn oil fed rats less of the limited acetate pool is required for fatty acid synthesis and thus, more is available for cholesterogenesis. Under other conditions, FOSTER AND BLOOM[11] have reported on a reciprocal relationship between fatty acid and cholesterol biosynthesis. Experiments designed to elucidate this relationship are in progress.

This work was supported, in part, by research grant HE 03299 and Career Award K6-HE-734 from the National Heart Institute.

Wistar Institute and School of Veterinary Medicine, University of Pennsylvania, and Veterans Administration Hospital and School of Dental Medicine, University of Pennsylvania, Philadelphia, Pa. (U.S.A.)

DAVID KRITCHEVSKY
JOSEPH L. RABINOWITZ

1 R. SCHOENHEIMER AND D. RITTENBERG, *J. Biol. Chem.*, 120 (1937) 155.
2 J. ELOVSON, *Acta Univ. Lund. Sect. II*, 14 (1965).
3 R. REISER, M. C. WILLIAMS, M. F. SORRELS AND N. L. MURTY, *Arch. Biochem. Biophys.*, 102 (1963) 276.
4 D. KRITCHEVSKY AND S. A. TEPPER, *J. Nutr.*, 86 (1965) 67.

5 E. F. PHARES AND M. V. LONG, *J. Am. Chem. Soc.*, 77 (1955) 2556.
6 J. L. RABINOWITZ, *Anal. Chem.*, 29 (1957) 982.
7 D. D. VAN SLYKE, J. PLAZIN AND J. R. WEISIGER, *J. Biol. Chem.*, 191 (1951) 299.
8 W. G. DAUBEN, E. HOERGER AND J. W. PETERSEN, *J. Am. Chem. Soc.*, 75 (1953) 2347.
9 J. AVIGAN AND D. STEINBERG, *Proc. Soc. Exptl. Biol. Med.*, 97 (1958) 814.
10 J. D. WOOD AND B. B. MIGICOVSKY, *Can. J. Biochem. Physiol.*, 36 (1958) 433.
11 D. W. FOSTER AND B. BLOOM, *Biochim. Biophys. Acta*, 70 (1963) 341.

Received October 18th, 1965

Proc. Soc. Exp. Biol. Med. 76: 741-743, 1951

Distribution of Radioactivity in the Egg after Feeding Sodium Acetate-1-C^{14} (18615)

DAVID KRITCHEVSKY, C. R. GRAU, B. M. TOLBERT AND BARBARA J. KRUCKEL.
(Introduced by John H. Lawrence)

From the Radiation Laboratory and the Division of Poultry Husbandry, University of California, Berkeley.

Earlier work on the distribution of radioactivity in eggs following the administration of radioactive isotopes of phosphorus, strontium and iron has been discussed in the com-

* This work was sponsored by the U. S. Atomic Energy Commission.

prehensive treatise of Romanoff and Romanoff (1). More recently, further work has been carried out using isotopic phosphorus(2) and calcium(3,4).

The present work was undertaken with the two-fold purpose of securing data to indicate the uptake of radioactive carbon in the egg and of obtaining from these eggs products of high enough specific activities to warrant their use in subsequent work. The isotopic carbon used was in the form of $CH_3C^{14}OONa$ and was mixed with the feed over a period of several days with a view to obtaining high activities in the eggs produced. The radioactive carbon was incorporated into the shell, albumen and yolk of every egg laid during the experimental period.

Experimental procedures. The sodium acetate-1-C^{14} (9.55 millicuries, 0.787 g, sp. act. 12.1 μc/mg) was prepared by carbonation of methyl magnesium iodide with $C^{14}O_2$ (5). The sodium acetate-1-C^{14} was dissolved in a small amount of water and the resulting solution was sprayed over the feed (a practical laying ration) which was being stirred by hand. After all the solution was added, the feed was thoroughly mixed. The labeled acetate was administered in a total of 3 feedings. The first feeding comprised 4.78 mc of acetate in 350 g of feed. Then 2.39 mc of acetate in 175 g of feed and 2.38 mc in 175 g of feed were fed on the sixth and eighth days, respectively, after the initial feeding. As soon as one portion of feed was exhausted, a new one was prepared and fed. Thus, the hen was fed labeled acetate for ten days, at which time she was sacrificed. The hen used was a Single Comb White Leghorn, maintained in a wire cage with a raised floor. The shells were washed free of adhering matter and the yolk and albumen of each egg was lyophilized separately. Each shell was freed of its shell membrane, which was lyophilized with the albumen, and then air dried and pulverized. Aliquots of the shell, yolk and albumen were converted by dry combustion to CO_2, which was collected and counted as barium carbonate. Seven eggs were collected over the 10 day period of the experiment. The eggs were collected on the second, third, fifth, sixth, eighth, ninth and tenth days after feeding was begun. The hen was sacrificed on the tenth day and the contents of the ovary were separated into 4 fractions comprising 2 large ova, a number of easily separated small ova and the ovary with the remaining ova. Each of these fractions was homogenized, lyophilized and the activity of an aliquot was determined.

Results and discussion. Romanoff and Romanoff(6) present data to show that the egg, in passing through the oviduct, spends 5 hours in the albumen secreting region and 19 hours in the uterus, where the shell is laid down. The fact that in egg 1 (which was laid within 24 hours after feeding was begun) radioactivity was present in the yolk, albumen and shell, reflects the very rapid metabolic utilization of the acetate.

In the first egg, most of the activity was found in the shell, which is understandable, since radioactive carbonate was being produced at the same time that carbonate was being utilized for shell formation. The specific activities of shells 2, 3, 5, 6 and 7 were roughly the same, the carbonate for these shells probably having been supplied by the metabolic pool. The activity of shell 4, formed soon after fresh feed was offered, was considerably higher.

The yolks exhibited a specific activity variation that parallels that of the shell, with one exception which will be noted shortly. The specific activities began to fall off as the feed was exhausted and had the hen been allowed to lay the 2 eggs represented by yolks 8 and 9, the yolk specific activities would have been 75 x 10^{-4} μc/mg and 36 x 10^{-4} μc/mg, respec-

1. Romanoff, A. L., and Romanoff, A. J., *The Avian Egg*, John Wiley and Sons, Inc., New York, 1949, p. 363.

2. O'Neil, J. B., Jowsey, R., Lee, C. C., Reade, M. V., and Spinks, J. W. T., *Science*, 1948, v107, 295.

3. Comar, C. L. and Driggers, J. C., *Ibid.*, 1949, v109, 282.

4. Spinks, J. W. T., Berlie, M. R., and O'Neil, J. B., *Ibid.*, 1949, v110, 352.

5. Calvin, M., *et al.*, *Isotopic Carbon*, John Wiley and Sons, Inc., New York, 1949, p. 178.

6. loc. cit., p. 223 ff.

TABLE I. Weights and Activities of Collected Eggs and Ova.*

Day	Acetate fed	Egg	Total activity of egg	Shell		Albumen		Yolk	
				Wt	Act.	Wt	Act.	Wt	Act.
1	4780								
2		1	6.4	6.50	4.8	3.35	0.2	8.56	1.4
3		2	53.0	6.22	16.9	3.27	3.8	8.61	32.3
5		3	57.3	6.95	16.1	4.40	8.3	8.81	32.9
6	2390	4	115.5	6.13	33.4	4.08	6.8	8.48	75.3
8	2380	5	56.4	6.35	20.6	4.30	11.3	8.30	24.5
9		6	132.7	6.11	13.7	3.54	8.1	8.37	110.9
10		7	129.6	5.00	10.7	4.21	5.8	9.33	113.6
—		Ovum 1	64.8	—	—	—	—	5.85	64.8
—		" 2	30.4	—	—	—	—	3.25	30.4
—		Ova	9.4	—	—	—	—	1.43	9.4
—		Ovary and ova	10.5	—	—	—	—	2.44	10.5

* All weights of lyophilized solid, given in g. All activities as μc.

tively, based upon average weight of yolks obtained.

Possible explanations for the low activity of yolk 5 are: (1) error in marking and perhaps in combustion of the yolk, or (2) failure of the hen to ovulate this yolk at the normal time, that is, to have produced an egg on the fourth day between eggs 2 and 3. No instances of such "held" yolks were found in the studies of Warren and Conrad(7). Further investigation into the specific activities of various yolk fractions will indicate which of these possibilities is the more valid.

The specific activity of all the albumen fractions was lower, which might be expected considering that for inclusion into albumen, the acetate fragment or its carboxyl moiety would be required to traverse longer metabolic pathways than for inclusion into shell or yolk. In general, a gradual increase of specific activity of the albumen fractions was observed, which would seem to indicate that the radioactive portion of this fraction was being supplied by the metabolic pool.

In all, 660 μc (0.96%) of the total fed activity was recovered in the eggs: of this, the yolks contained 495.5 μc (5.18%), the shells 116.2 μc (1.21%), the albumen 43.8 μc (0.46%) and the ovary and ova 10.5 μc (0.11%).

7. Warren, D. C. and Conrad, R. M., *J. Agr. Research*, 1939, x58, 875.

Summary. The data indicate that upon feeding of carboxyl-labeled sodium acetate, the activity is rapidly incorporated into all portions of the egg. The acetate is utilized immediately and a comparatively small percentage of radioactivity is diverted to the metabolic pool. Steady feeding of labeled material is required in order to maintain a high specific activity in all portions of the egg.

It is a pleasure to acknowledge the interest shown by Professors Melvin Calvin and Samuel Lepkovsky during the course of this work.

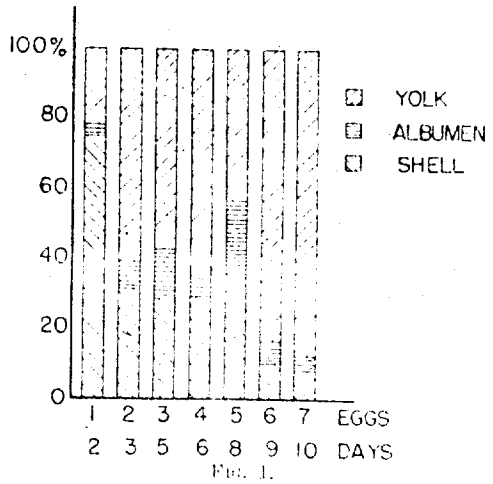


FIG. 1.
Distribution of total activity in laid eggs.

Received February 14, 1951. P.S.E.B.M., 1951, v76.

FURTHER OBSERVATIONS ON THE TERATOGENIC NATURE OF INSULIN AND ITS MODIFICATION BY SUPPLEMENTARY TREATMENT

WALTER LANDAUER AND MARION R. RHODES
University of Connecticut, Storrs

It was shown previously that insulin treatment of developing chicken embryos produces morphological abnormalities. These defects are chiefly, perhaps exclusively, skeletal, but what parts of the skeleton are affected depends on the stage during which the embryos had been exposed to the hormone. Incidence and degree of malformation vary with dose of injected insulin, hereditary constitution of the embryos, season, and probably numerous other and minor factors. Very high doses of insulin are sometimes responsible for the simultaneous occurrence of abnormalities which with smaller amounts only occur after treatment in other developmental stages. The fact that all insulin-induced malformations appear to be phenocopies of known mutants adds greatly to the interest of analyzing the physiological events by which insulin produces its effects. The more important results of our experiments have been reviewed in an earlier publication (Landauer, '48a). Since then we have found that the teratogenic consequences of insulin injection can be averted, in part or entirely, by supplementing insulin treatment with certain other substances, such as nicotinamide and α-ketoglutaric acid (Landauer, '48b). The present report deals with an extension of these experiments.

Nicotinamide

The injection of insulin into the yolk sac of chicken eggs during the 4th or 5th day of development is followed by a high

incidence of micromelia and beak defects. From the results of earlier experiments it appeared that the frequency of occurrence of micromelia could be much reduced by supplying nicotinamide along with insulin, but that such supplementation was less effective in regard to beak development. Survival of insulin-nicotinamide treated embryos was, however, less good in these experiments than in the group that had only been exposed to insulin, and, because of this some doubt remained concerning the validity of the conclusions drawn from these data. Hence, we decided to repeat the experiments. At the same time we desired to obtain information on the following two questions. Does nicotinamide actually protect the embryos less well against insulin-induced beak defects than against abnormalities of the extremities? Secondly, will nicotinamide supplementation forestall the phenotypic effects that insulin has on hereditary polydactylism (Landauer, '48c)?

For our new experiments on the combined use of insulin (Iletin, Lilly) and nicotinamide (Niacinamide, Squibb) we used eggs from a mating of White Leghorn females to a Dorking male, the latter being homozygous for polydactylism. The results for incidence of micromelia and beak defects are given in table 1. Each of the three experimental groups included one lot in which insulin alone, in a dose of either two units (A and B) or one unit (C), was injected at 90 hours of development and another lot that served as untreated control. In addition, one lot of group A received two units of insulin and 5 mg nicotinamide at 93 hours, one of group B was given 10 mg of nicotinamide at 93 hours, followed by two units insulin at 96 hours, and one lot of group C was injected with 10 mg nicotinamide at 93 hours and one unit insulin at 96 hours. The results are unequivocal. When 5 mg nicotinamide were given simultaneously with two units insulin, the incidence of all types of malformations was much reduced; when the embryos were supplied with 10 mg of nicotinamide three hours prior to the injection of either one or two units of insulin, protection against the teratogenic ef-

fects of insulin was complete. In none of the three groups had nicotinamide a statistically significant effect on embryo mortality due to injection of insulin. In other tests, the results of which are not reproduced here, we found that higher doses of nicotinamide (20 mg) — similar in amount to those

TABLE 1

Experiments concerning the effect of nicotinamide on insulin-induced abnormalities of extremities, beak and eyes

F_1 embryos from Leghorn ♀♀ × Dorking ♂

GROUP	TREATMENT	MORTALITY PRIOR TO 16TH DAY	SURVIVORS OF 16TH DAY	MICROMELIA	BEAK ABNORMALITIES	EYE ABNORMALITIES
		%		%	%	%
A	Insulin 2 U. — 96 hours	26.8	60	45.3	23.8	6.7
	Insulin 2 U. + 5 mg nicotinamide — 96 hours	13.6	70	10.0	4.3	1.4
	Untreated	3.7	79	0	0	0
B	Insulin 2 U. — 96 hours	12.9	74	55.4	29.7	1.4
	Nicotinamide 10 mg — 96 hours, insulin 2 U. — 96 hours	19.5	63	1.5	0	0
	Untreated	10.0	72	1.4	0	0
C	Insulin 1 U. — 96 hours	24.4	96	16.7	9.4	2.1
	Nicotinamide 10 mg — 96 hours, insulin 1 U. — 96 hours	25.1	103	0	1.0	0
	Untreated	10.7	117	0	0	0

used in our earlier experiments — were definitely toxic whether used in conjunction with insulin or alone, but among 33 survivors only a few showed micromelia and none had beak defects.

It is clear from these results that the occurrence of those effects of insulin treatment (during the 4th or 5th day of incubation) which resemble the phenotypes of major gene

changes, viz. micromelia and beak defects, can be prevented by adequate and properly timed supplementation with nicotinamide. We have reported earlier that in polydactylous stocks insulin has modifier-like effects in regard to development of the toes. Data on the question whether or not these effects can be prevented by nicotinamide supplementation appear in table 2. These observations must be reviewed with reference to the different modes of expression of polydactylism. In the first place, it will be seen that, as reported earlier, insulin treatment greatly exaggerated the incidence of phenotypic lack of expression of the gene for polydactylism (column A). This insulin effect was not significantly altered when 5 mg nicotinamide were given together with two units insulin. When, however, 10 mg nicotinamide were injected three hours prior to either one or two units insulin the occurrence of non-polydactylous embryos was reduced very significantly ($\chi^2 = 9.90$ with $P = .002$ in the first instance and $\chi^2 = 11.71$ with $P = < .001$ in the second). As for the different modes of expression of polydactylism, it can be seen that under the influence of nicotinamide-supplementation there was a definite trend toward an increased incidence of the irregular types, such as heterodactylism and polyphalangism (E and F), in preference to a return to the bilaterally 5-toed condition which is typical for the control embryos. With optimal amounts of nicotinamide, however, the incidence of typical bilateral polydactylism was also augmented conspicuously (C). It is clear, therefore, that the changes which insulin produces in the expression of polydactylism can to some extent be prevented by nicotinamide-supplementation, but that the protective effect is lower than with reference to micromelia and beak abnormalities. This response difference will be discussed later.

In an earlier publication (Landauer, '48b) we have reported that a significant reduction in incidence of rumplessness was obtained when injection of two units insulin at 24 hours was supplemented with 18.9 mg nicotinamide, but that nicotinamide did not at this stage give the embryos as much

TABLE 2

Experiments concerning the effect of nicotinamide on insulin-induced modifications of polydactylism[1]

(This is the same material as in table 1)

F_1 embryos from Leghorn ♀♀ × Dorking ♂

TREATMENT	SURVIVORS OF 17TH DAY	A NON-POLY-DACTYLOUS	B BILATERAL POLY-DACTYLISM	C TYPICAL, BUT WITH CONDITION ON FOUR FEET	D ATYPICAL BILATERAL POLY-DACTYLISM	E BILATERAL POLY-DACTYLISM	F BIPED-DACTYLISM
		%	%	%	%	%	%
Insulin 2 U. — 96 hours	60	68.3	33.3	18.3	15.0	1.7	0.7
Insulin 2 U. — 6 mg nicotinamide — 96 hours	70	65.7	28.6	17.1	11.5	4.8	11.4
Untreated	79	12.7	77.2	62.0	15.2	2.5	7.6
Insulin 2 U. — 96 hours	74	62.2	28.4	14.9	13.5	1.4	8.1
Nicotinamide 10 mg — 93 hours, insulin 2 U. — 96 hours	60	33.3	46.6	29.8	16.7	6.1	15.2
Untreated	72	11.1	76.1	62.5	13.9	4.2	8.3
Insulin 1 U. — 96 hours	96	40.6	40.6	25.0	15.6	5.2	13.5
Nicotinamide 10 mg — 93 hours, insulin 1 U. — 96 hours	100	20.0	46.0	33.0	14.0	11.0	25.0
Untreated	117	11.1	72.6	63.2	9.4	6.9	9.4

[1] Columns A, B, E and F contain the total of all observations; the data of B are broken down in C and D.

protection against insulin damage as it does at later stages (96 and 120 hours). Since the amount of nicotinamide used in these experiments proved to have considerable toxicity, we decided to repeat the experiment with a smaller amount of nicotinamide, viz. 5 mg. The results are summarized in table 3. The nicotinamide was injected three hours prior to insulin, simultaneously with it, or 3, 6 and 12 hours after insulin. Embryo mortality was, even with this lower dose of nicotinamide, much increased. When nicotinamide was given between three hours prior to and 6 hours following insulin treatment, the incidence of rumplessness was in each instance lower than with insulin alone. Corresponding to the smaller dose of nicotinamide, its effectiveness in forestalling rumplessness was reduced, and the differences were in no case great enough to be statistically significant. However, the χ^2 from the combination of probabilities for groups 2 to 5 as against group

TABLE 3

Experiments concerning the effect of nicotinamide on the occurrence of insulin-induced rumplessness in White Leghorn embryos

Dosage: 2 units insulin, 5 mg nicotinamide

GROUP	TREATMENT	FERTILE EGGS	MORTALITY PRIOR 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESS (ALL TYPES)	ABNORMAL BEAK
			%		%	%
1	Insulin — 24 hours	200	39.5	121	26.4	0
2	Nicotinamide — 24 hours insulin — 27 hours	316	54.4	127	20.1	2.1
3	Insulin and nicotinamide 24 hours	347	62.8	119	21.0	5.9
4	Insulin — 24 hours nicotinamide — 27 hours	343	63.5	90	15.6	5.6
5	Insulin — 24 hours nicotinamide — 30 hours	259	54.3	138	15.2	3.6
6	Insulin — 24 hours nicotinamide — 36 hours	233	54.4	122	28.8	8.3

1 was highly significant ($\chi^2 = 19.933$, $df = 8$, $P = .01$). Nicotinamide given 12 hours later than insulin no longer affected the incidence of rumplessness. On the whole, these results confirm with a lower dosage (viz. 5 mg) our earlier conclusion that an amount of nicotinamide which completely or nearly completely forestalled the micromelia-inducing effects of two units insulin at 96 or 120 hours of incubation, reduced only slightly the incidence of rumplessness after two units insulin at 24 hours and that the toxicity of this amount of nicotinamide was much greater at the earlier period than at the later one.

Some observations on beak defects, however, do not seem to fit into the general interpretation of the protective action of nicotinamide. In experiments with sulfanilamide, Zwilling and DeBell ('50) found that nicotinamide-supplementation completely forestalled the appearance of micromelia. But the parrot-beak condition that generally accompanies sulfanilamide-induced micromelia was, in the presence of nicotinamide, frequently replaced by a shortening of the upper beak. This effect of nicotinamide-supplementation was found when both sulfanilamide and nicotinamide were given at the end of the 5th day of incubation, but also when sulfanilamide was injected at 120 hours and nicotinamide 24 or even 48 hours later. We had observed earlier (Landauer, '48b) that nicotinamide supplementation of insulin treatment at 24 hours was responsible for the appearance of a certain proportion of embryos with shortened upper beaks. Our present results bear out these observations. We had suggested earlier that "a shift of the insulin damage within embryos" had been produced by nicotinamide. This interpretation is no longer tenable since it was found that nicotinamide by itself will in certain developmental stages call forth abnormalities of the beak. This is true for 24-hour embryos, but does not hold at all or to only a very slight extent at 96 hours (table 4). The observations of Zwilling and DeBell, to which we referred, suggest, however, that responses similar to those at 24 hours may occur again after 5 to 7 days of development.

Glucose-1-phosphate, citric and oxalacetic acid

Experiments in which insulin treatment at 96 hours was combined with the injection of glucose-1-phosphate produced results of considerable interest (table 5). Glucose-1-phosphate by itself was non-toxic and without teratogenic effects. When the injection of two units of insulin was supplemented with 4 mg glucose-1-phosphate the post-operative mortality was higher than after insulin alone; there was no significant

TABLE 4

Effect of nicotinamide, injected at 24 or 96 hours of incubation, on rate of mortality and beak development. Eggs from White Leghorn fowl

		Age at injection 24 hours Dose 5 mg	Age at injection 96 hours Dose 5 mg	Age at injection 96 hours Dose 10 mg	Significance of differences between injections at 24 and 96 hours for dose of 5 mg
Number of fertile eggs injected		202	128	122	
Mortality during first 6 days and from 96 hours to end of 2nd week, respectively	%	16.1	2.1	8.1	$\chi^2 = 13.949$ $P = <.001$
Survivors of 17th day		225	117	110	
Short upper beak	%	7.2	0.9	1.8	$\chi^2 = 5.336$ $P = <.02$
Other abnormalities		0	0	0	

change in the incidence of micromelia or syndactylism (the latter not shown in the table) among the embryos surviving the 17th day of development, but the frequency of beak defects was greatly reduced. The experiment was performed twice with similar results. It will be noted that the incidence of micromelia and of beak abnormalities was considerably lower in the second experiment than in the first. This was true whether insulin was used alone or in combination with glu-

TABLE 5

The effect of supplementation of insulin-treatment with glucose-1-phosphate, citric acid and oxalacetic acid at 96 hours of incubation. White Leghorn eggs

Dosage of Insulin 2 units throughout

DATE	TREATMENT	NUMBER OF TREATED EMBRYOS	MORTALITY FROM 96 HOURS TO END OF 2ND WEEK	SURVIVORS OF 17TH DAY	MICRO-MELIA	APPARENT BEAK	OTHER ANOM-ALIES	COMPARISON OF PERCENTAGES OF BEAK ANOMALIES
			%		%	%	%	
4/25	Insulin + 4 mg glucose-1-phosphate	140	48.0	61	78.1	9.4	6.3	$\chi^2 = 7.006$ $P = <.01$
	Insulin	167	51.0	112	76.0	20.8	4.5	
5/30	Insulin + 4 mg glucose-1-phosphate	307	36.8	189	61.3	16.4	2.0	$\chi^2 = 15.078$ $P = <.0001$
	Insulin	153	28.8	60	48.0	37.5	6.9	
	Insulin + 25 mg citric acid	162	43.2	60	61.1	14.9	8.0	$\chi^2 = 15.017$ $P = <.0001$
	Insulin + 7.15 mg oxalacetic acid	150	42.0	63	37.3	11.5	5.1	$\chi^2 = 12.142$ $P = <.0001$

cose-1-phosphate and is an expression of a seasonal trend, already noted in earlier experiments (Landauer, '45). The relative reduction in frequency of beak defects under the influence of glucose-1-phosphate supplementation was, however, of a similar order in the two experiments. The beak abnormalities which occurred were of 4 different kinds, viz., parrot beak, shortened upper beak, cleft palate and cross-beak. Parrot beak is the only one of these 4 types of defects which, in its milder forms of expression, might lead to mistaken classification. It was, therefore, decided to scrutinize the effect of glucose-1-phosphate by adding all specimens with parrot beak to the normal groups, thus limiting the statistical analysis to those forms of abnormality in which we were dealing with presence or absence. Even with this, unquestionably over-stringent, procedure it was clear that glucose-1-phosphate furnished protection against insulin-induced beak abnormalities ($\chi^2 = 10.645$; $P = .001$).

A similar lowering in the incidence of beak defects was observed when insulin-treatment was supplemented with fructose-6-phosphate (5 mg/egg), but our data are too scanty to be trusted without reserve. Supplementation of insulin-treatment at 96 hours with fructose-1,6-diphosphate, on the other hand, clearly did not modify any of the teratogenic effects of insulin.

At 24 hours, supplementation of insulin with glucose-1-phosphate (4 mg/egg), fructose-6-phosphate (5 mg/egg) or fructose-1,6-diphosphate (2.7 mg/egg) had no significant effect on subsequent incidence of rumplessness. In two tests with glucose-1-phosphate we observed, however, that embryo mortality was increased significantly and micromelia was found in 10 and 11%, respectively, of the survivors of the 17th day. There were no cases of micromelia among the embryos treated with insulin alone.

Results similar to those with glucose-1-phosphate were obtained when insulin-treatment at 96 hours was combined with the injection of oxalacetic or citric acid. Embryo mortality was increased by these supplements, the frequency with which

[illegible] occurred was not affected, but the incidence of [illegible] was increased to a highly significant extent (table [illegible]). When given alone, oxaloacetic acid had appreciable toxicity [illegible] (mortality to end of second week [illegible]), but [illegible] was harmless.

At the 24-hour stage, supplementation with oxaloacetic acid did not significantly change the incidence of insulin-induced rumplessness. Citric acid, on the other hand, greatly reduced embryo mortality and incidence of rumplessness when added to insulin treatment at 24 hours (table 6). The results are

TABLE 6

The effect of citric acid on insulin-induced rumplessness. White Leghorn embryos. All injections at 24 hours

Dosage: insulin 2 units, citric acid 25 mg

TREATMENT	FERTILE EGGS	MORTALITY FIRST 6 DAYS	SURVIVORS OF 12TH DAY	RUMPLESS (ALL TYPES)	OTHER ANOMALIES
		%		%	%
Insulin	165	52.1	65	29.2	9.2
Insulin + citric acid	171	17.0	135	11.1	2.9
Citric acid	165	2.6	157	1.3	0

highly significant in both respects (χ^2 for embryo mortality = 43.035, P .0001; for incidence of rumplessness χ^2 = 10.165, P = .0035) and comparable to those obtained with lactic acid which will be discussed below. Isocitric acid (2.5 mg) given with insulin at 24 hours led to increased mortality and potentiation of the insulin effect (44.7% rumplessness, 10.5% both abnormalities).

Pyruvic and lactic acid; sodium acetate

In testing for the alleviatory effect of other compounds on the teratogenic action of insulin we were particularly interested in relatively simple metabolic intermediates such as

pyruvic, lactic and acetic acid. Our observations with undiluted pyruvic acid (Matheson Co.) are summarized in tables 7 and 8. It can be seen that pyruvic acid, when given simultaneously with insulin, is very effective in forestalling the occurrence of rumplessness (table 7). The combined administration of these two substances at 24 hours of incubation reduced the incidence of rumplessness, in comparison to that after unsupplemented insulin treatment, from 29.1% to

TABLE 7

Experiments concerning the effect of pyruvic acid on insulin-induced rumplessness in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ undiluted pyruvic acid

GROUP	TREATMENT	FERTILE EGGS	MORTALITY FIRST 6 DAYS	SURVIVORS OF 12TH DAY	RUMPLESS
			%		%
1	Insulin — 24 hours	254	45.3	139	29.1
2	Insulin and pyruvic acid — 24 hours	250	18.8	174	5.2
3	Insulin — 24 hours pyruvic acid — 27 hours	243	39.1	124	16.9
4	Insulin — 24 hours pyruvic acid — 30 hours	257	40.1	134	17.2
5	Insulin — 24 hours pyruvic acid — 36 hours	245	41.6	124	19.4
6	Pyruvic acid — 24 hours	171	10.5	146	2.7

5.2% (χ^2 = 21.252; P<.0001), and embryonic mortality during the first 6 days dropped from 45.3% to 18.8% (χ^2 = 47.206; P<.0001). If pyruvic acid is, however, supplied three or more hours subsequent to the injection of insulin, it has little, if any, beneficial effect on either incidence of rumplessness or toxicity of the insulin. No similarly beneficial results followed phosphopyruvic-acid supplementation (2 mg/egg); in fact, the toxicity in combination with insulin was very high and the incidence of rumplessness increased.

TABLE 8

Experiments concerning the effect of pyruvic acid on insulin-induced micrognathia and beak defects in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ [illegible] pyruvic acid

GROUP	TREATMENT	NUMBER OF TREATED EMBRYOS	MORTALITY TO END OF 2ND WEEK	NUMBER OF SURVIVORS PER DAY	MICROGNATHIA	ABNORMAL BEAK	[illegible] BEAK
			%		%	%	%
1	Pyruvic acid — 117 hours Insulin — 120 hours	403	71.0	74	73.7	29.7	5.4
2	Insulin and pyruvic acid — 120 hours	436	69.8	77	71.4	31.7	2.6
3	Insulin — 120 hours	225	20.0	103	33.9	4.9	0.9
4	Insulin — 120 hours pyruvic acid — 123 hours	393	77.8	62	75.0	12.5	0
5	Pyruvic acid — 120 hours	137	73.9	33	0	0	0

The effect of pyruvic-acid supplementation is very different at the time when insulin treatment leads to the occurrence of micrognathia and beak defects (table 8). Pyruvic acid, given simultaneously with insulin-injection or within three hours prior or subsequent to it, greatly increases, at this stage of development, embryo mortality as well as incidence of micrognathia and beak defects. All differences in these respects between group 3 (insulin alone) and the other three groups are statistically highly significant, except for that relating to the incidence of beak abnormalities in groups 2 and 3. The mortality

TABLE 9

Experiments concerning the effect of lactic acid on insulin-induced [illegible] in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ lactic acid ([illegible])

GROUP	TREATMENT	FERTILE EGGS	MORTALITY TO END OF 2 DAYS	[illegible]	[illegible]
			%		%
1	Insulin — 24 hours	336	49.0	101	[illegible]
2	Insulin and lactic acid — 24 hours	334	20.4	[illegible]	[illegible]
3	Insulin and lactic acid — 24 hours	325	10.8	[illegible]	10.1
4	Insulin and lactic acid — 24 hours	368	15.2	[illegible]	[illegible]

which was encountered in these experiments appears to be wholly due to the pyruvic acid. When pyruvic acid was injected by itself at 120 hours, mortality to the end of the second week amounted to 83.2% out of 137 treated embryos. No abnormalities of any kind were, however, found among the survivors. Phosphorpyruvic acid, given by itself, was harmless at this stage. In combination with insulin it increased embryo mortality without changing the incidence of abnormalities.

The results of experiments with lactic acid are shown in tables 9 and 10. When insulin treatment at 24 hours was

TABLE 10

Experiments concerning the effect of dl-lactic acid on lactic-induced micromelia and beak abnormalities in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ lactic acid (10%)

Group	Treatment	Number of eggs incubated	Percentage of eggs dead after 216 hours	Number of eggs dead	Micromelia	Abnormal beak	Abnormal beak and micromelia
			%		%	%	%
1	Lactic acid — 117 hours Insulin — 120 hours	234	44.0	120	84.0	6.8	0
2	Insulin and lactic acid — 120 hours	221	60.7	78	41.0	12.8	1.3
3	Insulin — 120 hours	220	50.4	220	51.6	11.0	0.0
4	Insulin — 120 hours lactic acid — 120 hours	228	60.0	111	40.5	8.1	0.0
5	Lactic acid — 120 hours	148	84.7	97	0	0	0

supplemented by injection of racemic lactic acid, the incidence of rumplessness and embryo mortality were reduced to about one-half of that occurring after treatment with insulin alone. The differences are in both instances highly significant (between groups 1 and 2 χ^2 for embryo mortality was 36.451 with $P<.0001$, and for incidence of rumplessness $\chi^2 = 9.312$; $P<.01$). A repetition of the experiment with dl-lactic acid gave closely similar results (group 4), but with d-lactic acid (Pfanstiehl), the naturally occurring form, the results were less favorable than with the racemic acid.

The results of lactic-acid supplementation of insulin-treatment at 120 hours are shown in table 10. When racemic lactic acid was given three hours prior to insulin injection, the incidence of micromelia was reduced significantly ($\chi^2 = 8.708$, $P<.01$); there were also fewer beak abnormalities, but in this instance the difference was not statistically significant. Injection of lactic acid simultaneously with or three hours subsequent to insulin-treatment had no significant effects on incidence of either micromelia or beak abnormalities. It can be seen, on the other hand, that the combined treatment with lactic acid and insulin produced in all groups increased embryo mortality (in all instances $P<.0001$). This is accounted for by the high degree of toxicity which lactic acid alone has at this stage of development (group 5). As was true for pyruvic acid, lactic acid reduced the mortality from insulin treatment in early embryonic stages, but had a highly toxic effect in later ones.

Sodium acetate in a dose of 10 mg/egg was toxic, without producing abnormalities, but when injected together with insulin resulted in an exaggerated incidence of the teratologic effects of insulin as well as heightened embryo mortality. This was especially true at 24 hours, but to a lesser extent also at 96 hours.

Amino acids

We performed some experiments with l-tryptophane (1.2 mg/egg), dl-methionine hydrochloride (3 mg/egg), l-glutamic

acid hydrochloride (12.5 and 25 mg/egg) and dl-alanine (7.2 mg/egg). When administered by themselves and in the given amounts, none of these amino acids, with the possible exception of l-glutamic acid hydrochloride, was teratogenic at either 24 or 96 hours of development. Except for l-glutamic acid hydrochloride, no striking toxicity was observed after injection of these compounds. At 24 hours, l-glutamic acid hydrochloride was somewhat toxic and pronouncedly so at 96 hours. Methionine, tryptophane and alanine in combination with insulin led to increased embryo mortality and among the survivors the incidence of rumplessness was higher than after insulin alone. At 96 hours these three amino acids probably produced no significant change in embryo mortality, nor in the teratogenic effects of insulin. A more interesting situation obtains in regard to supplementation with l-glutamic acid hydrochloride. Injected alone, this compound showed some toxicity for 24-hour embryos (19.5 ± 3.45% mortality during first 6 days) and probably had a slight rumplessness-inducing effect (6.5 ± 2.46%). We made three tests at 24 hours in which the injection of two units insulin was supplemented by 12.5 mg/egg l-glutamic acid hydrochloride and two tests in which 25 mg/egg of this substance were given in addition to insulin. Since there was no apparent inconsistency between the results for the two different dosage levels, we are here reproducing only the totals for all 5 tests (table 11). It can be seen that l-glutamic acid hydrochloride supplementation reduced mortality as well as incidence of rumplessness. At 96 hours l-glutamic acid hydrochloride supplementation sharply increased embryo mortality, but did not change the teratogenic action of insulin.

Sodium succinate and 3-hydroxy-anthranilic acid

Some substances which are toxic and growth-retarding, but do not by themselves produce any teratological results, potentiate strikingly the incidence of insulin-induced micromelia and beak abnormalities when given together with in-

sulin at 96 or 120 hours of development. This is true, for instance, for adrenal cortex extract (Landauer, '47a). There are other substances which produce a similar potentiation of insulin action, but, when given alone and in the same amounts, neither affect viability nor interfere with normal development. Among compounds of the latter type are sodium succinate and 3-hydroxy-anthranilic acid. In experiments with 96-hour embryos 91.9% of all embryos survived the 17th day after the injection of 4 mg 3-hydroxy-anthranilic acid; among the 147 survivors the only abnormalities found were two cases of micromelia and one of cleft palate. After the injection of 8 mg

TABLE 11

Results of 4 tests with insulin alone and in combination with l-glutamic acid hydrochloride at 24 hours of development. Two units insulin all tests; l-glutamic acid hydrochloride 12.5 and 25 mg/egg, respectively, in different tests. White Leghorn eggs. Standard errors.

TREATMENT	FERTILE EGGS	MORTALITY DURING FIRST 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESSNESS
		%		%
Insulin	667	37.0 ± 1.61	391	24.3 ± 2.15
Insulin + l-glutamic acid hydrochloride	531	24.8 ± 1.50	600	13.3 ± 1.38
Difference		12.2 ± 2.43		11.0 ± 2.55

sodium succinate 83.8% of all embryos survived the 17th day and among the 143 survivors one rumpless chick was the only abnormality observed. When the same amounts of sodium succinate or 3-hydroxy-anthranilic acid were injected simultaneously with insulin, we obtained the results shown in table 12. For comparison we give the data for 4 tests with insulin alone, done during the same period. It can be seen that the incidence of all malformations was greatly exaggerated by the presence of either sodium succinate or 3-hydroxy-anthranilic acid. This potentiating effect was relatively the greater, the lower was the frequency with which a particular type of abnormality occurred under the influence of unsupplemented

TABLE 12

Data concerning the potentiating effect of sodium succinate and 3-hydroxy-anthranilic acid on the teratogenic action of 2 units insulin at 96 hours.

White Leghorn eggs

TREATMENT		SURVIVORS OF 17TH DAY	MICROMELIA	SYNDACTYLISM	BEAK ABNORMALITIES
			%	%	%
Insulin + 8 mg sodium succinate 5/23/50		65	84.2 ± 2.75	18.9 ± 4.02	34.7 ± 3.17
Insulin + 4 mg 3-hydroxy-anthranilic acid 6/23/50		65	89.2 ± 3.86	36.9 ± 6.0	69.2 ± 5.75
Insulin (4 tests) 5/23 to 6/27 1950	Mean	425[1]	60.0 ± 2.38	4.5 ± 1.01	26.1 ± 2.13
	Range	96–123	49.0–66.3	2.4–6.3	19.2–27.5

[1] Total of the 4 experiments.

insulin injection. For combined treatment with insulin and 3-hydroxy-anthranilic acid the degree of potentiation in terms of the mean incidence in the unsupplemented insulin control groups is demonstrated by the following figures.

	INCIDENCE AFTER INSULIN ALONE	POTENTIATION DUE TO 3-HYDROXY-ANTHRANILIC ACID
	%	%
Micromelia	60.0	48.7
Beak defects	26.1	165.1
Syndactylism	4.5	720.0

Syndactylism shows the most extreme effect. Syndactylism, not heretofore reported as an insulin-induced abnormality and to be discussed in more detail subsequently, was found in about 4.5% of Leghorn embryos which had been treated with two units of insulin at 96 hours. When, in addition to insulin, 8 mg of sodium succinate or 4 mg of 3-hydroxy-anthranilic acid were injected, the incidence of syndactylism rose to 18.9% and 36.9%, respectively.

Results with sodium succinate at 24 hours were very similar to those at 96 hours. Given by itself, it was non-toxic and non-teratogenic; added to insulin treatment, it greatly increased embryo mortality and incidence of rumplessness. The effect of 3-hydroxy-anthranilic acid at 24 hours was more complex. When this compound was injected by itself (4 mg/egg), it showed some toxicity (mortality during first 6 days 29.5 ± 3.5%) and among the survivors of the 17th day we found 11.9% rumpless embryos. In a preliminary test with the same dosage the frequency of rumplessness had been 19% and for the combined data we have an incidence of 11.4 ± 2.59%. It is evident that 3-hydroxy-anthranilic acid is by itself relatively potent in inducing rumplessness. Combined injection of insulin and 3-hydroxy-anthranilic acid resulted in very high mortality (84.4% during the first 6 days), but among the small number of survivors the incidence of rumplessness was not remarkably high (18.2%), possibly on account of differential mortality. On the other hand, this treatment led to the occurrence of some cases of micromelia, cleft palate and syndactylism, none of which were at this developmental stage produced by two units of insulin alone.

DISCUSSION

The major results of our experiments are as follows. A repetition and extension of earlier experiments with nicotinamide showed anew that, if proper dosage levels were used and the amount of administered insulin was not too great, supplementation with this vitamin gave complete protection against the usual teratogenic effects of insulin treatment at 96 or 120 hours of incubation. In spite of the fact, however, that supplementary nicotinamide prevented the occurrence of micromelia and beak defects[1] among the survivors to late stages, the post-operative mortality was not reduced. To a certain extent nicotinamide also protected genetically polydactylous embryos against the modifier-like effects that in-

[1] These malformations of the beak have nothing in common with the post-mortem beak abnormalities reported by Blattner and Williamson ('50).

sulin has on the expression of this mutation. Lactic acid, when given three hours prior to insulin treatment, reduced the incidence of micromelia, but had little influence on the frequency of beak abnormalities. Lactic acid had no protective value when administered simultaneously with or subsequent to insulin. In all these situations the combined use of insulin and lactic acid was, however, highly toxic. In contradistinction to the effects of lactic acid, supplementation of insulin-treatment with citric acid (when given simultaneously) did not change the incidence of micromelia, but furnished considerable protection against the occurrence of beak defects. Results similar to those with citric acid were obtained with glucose-1-phosphate, oxalacetic acid and probably fructose-6-phosphate.

In accord with earlier observations we found that during the rumplessness-inducing period of insulin (24 hours of incubation) nicotinamide gave some protection to embryos, but that its effectiveness was much lower than at the later stage and that, in addition, it was definitely toxic at this earlier time. Supplementary pyruvic acid, on the other hand, sharply reduced the incidence of rumplessness and greatly lowered the toxicity which follows treatment with insulin alone. Among many substances tested, pyruvic acid was most effective in this respect. Results of a similar nature, but at a lower level of efficiency, followed supplementation with lactic and citric acid; l-glutamic acid hydrochloride gave a slight, but significant, amount of protection. From present and earlier evidence we may conclude that the sequence of protection against insulin-induced rumplessness, beginning with the most effective compound, is about as follows: pyruvic acid > lactic acid = citric acid > nicotinamide > α-ketoglutaric acid ≥ l-glutamic acid hydrochloride.

It has been shown in previous communications that the production of micromelia and beak defects can be achieved by treatment with eserine, sulfanilamide or insulin and that in all three instances supplementary nicotinamide protected embryos against the teratogenic action of the three compounds

(Landauer, '49; Zwilling and DeBell, '50). We could further demonstrate that the rumplessness-inducing action of sodium cacodylate and insulin can similarly and to a large extent be forestalled by added pyruvic acid. It seems justified to draw three conclusions from this evidence, viz., (1) that, as pointed out by Ancel ('45b, '50), certain chemical compounds have more or less specific teratogenic potentialities; (2) that insulin has several different potentialities depending on developmental stage; and (3) that, irrespective of the teratogenic agent employed, the same supplements were effective in forestalling the occurrence of a particular malformation or in reducing its incidence.

There is additional evidence in support of these conclusions. The two substances that in Ancel's ('45a, '50) experiments most regularly produced micromelia and beak defects, viz., eserine and sulfanilamide, were also the ones that led most frequently to the occurrence of syndactylism. We had not previously reported on the production of syndactylism by insulin, but our present observations show that during the stages when insulin treatment leads to the occurrence of micromelia and malformations of the beak, it is also responsible for the appearance of a certain incidence of syndactylism. Nicotinamide protects the embryo against this abnormality as well as against the other symptoms of insulin-treatment typical for the 96- or 120-hour stages. Syndactylism thus represents another malformation that can be produced by three different and unrelated chemical compounds and that in all instances can be forestalled by the same supplement. It is of particular interest to note that insulin-induced syndactylism always involves the 3rd and 4th toes, as does hereditary syndactylism. This is additional evidence for our earlier conclusion that the majority, perhaps all, of the insulin-induced abnormalities are of the nature of skeletal phenocopies. Hereditary syndactylism was believed to be inseparable from brachydactylism and feathering of the feet (Danforth, '19; Jaap, '39), but no instances of such association occurred in our material and Warren ('50) has recently

described a hereditary form of syndactylism, unaccompanied by these traits.

Among the beak defects produced by insulin one can distinguish 4 different types, viz., parrot beak (associated with a moderate shortening of the lower beak), short upper beak, cross-beak and cleft palate. The proportions in which these defects occur vary considerably from experiment to experiment, probably parallel with minor differences in the developmental stages obtaining at the time of treatment. All 4 types of beak defects are prevented by supplementary nicotinamide. We had illustrated a case of cleft palate and facial coloboma in an earlier publication (Landauer, '47b, fig. 4) but did not then describe it in detail. Ancel could produce cleft palate with trypaflavin, but with none of the other substances tested by him, in particular not with eserine and sulfanilamide, the two compounds leading to a high incidence of micromelia and parrot beak. This is, therefore, another instance in which insulin has the greater teratogenic latitude and in which it has been shown that nicotinamide-preventable abnormalities may be separately produced by unrelated chemical compounds. It cannot be doubted that these various substances, producing the same malformations and being made non-teratogenic by the same supplements, create their effects via identical or closely similar pathways.[2]

Throughout our observations it has become evident, however, and we shall have further occasion to discuss it, that, in spite of these similarities in the teratogenic action of groups of substances, there are many minor, but significant, dissimilarities in their effects. We shall here call attention to two examples concerning the position in time of the maximum susceptible period for certain compounds. It was shown earlier that the incidence of rumplessness after insulin treat-

[2] In mammalian embryos cleft palate has been produced experimentally in rats with trypan blue (Gilmann, Gilbert and Gillman, '48), maternal riboflavin deficiency (Warkany, Nelson and Schraffenberger, '43), and maternal folic acid deficiency (Evans, Nelson and Asling, '51), in mice as a consequence of anoxia (Ingalls, Curley and Prindle, '50). Some of these techniques may in their biochemical effects be related to those described for chicken embryos.

ment increases from 0 hours (unincubated eggs) to a maximum early in the second day of incubation (Landauer and Bliss, '46). In contradistinction, sodium cacodylate has a significantly higher rumplessness-inducing effect at 0 hours than later. It is also of interest that following treatment with sodium cacodylate the protective value of nicotinamide seems to decrease during the same period (0–24 hours). A difference similar to that between sodium cacodylate and insulin in regard to the production of rumplessness, exists between sulfanilamide and insulin with reference to the origin of micromelia. The incidence of micromelia after insulin-treatment rises to a maximum at approximately 120 hours of incubation (Landauer and Bliss, '46), whereas sulfanilamide-induced micromelia reaches its peak frequency at about 48 hours with a steep decline thereafter, the incidence following injection at 120 days being only about one-quarter that at 48 hours (Zwilling and DeBell, '50). In spite of these great differences in the stages of maximum effectiveness, nicotinamide completely forestalls the occurrence of micromelia in both instances. It is difficult to escape the conclusion that these time dissimilarities of chemical action are caused by effects on different, if closely related, links of the same metabolic chain, the links in question presumably having varying biochemical importance at one time or another, but damage to the chain being in either event repairable by identical supplements.

Of apparently quite another order are the events that, at different developmental stages, produce distinctive and dissimilar abnormalities, such as rumplessness or micromelia. Attention has already been called to the fact that, according to Ancel's observations, some chemicals have "specific" teratogenic effects. It does not reduce the importance of this finding that in those instances where well-defined stage-response differences exist, as with insulin, the stage "specificity" can to some extent be overcome by an increased dose of the teratogenic agent or by supplementation with substances that by themselves are non-teratogenic. For, even

with such substances as insulin. It is clear that the response differences of the embryo in different developmental stages are not merely the effect of an interference with identical physiological processes in altered morphological situations.

There is evidence of a varied nature for the conclusion that the origins of these stage and type-specific effects are separated by important changes in biochemical functions. To start with, it has been seen that striking differences exist in the protective efficiency of various supplements, such as pyruvic acid being best in forestalling rumplessness, irrespective of the chemical character of the teratogenic agent, and nicotinamide being the most successful protective substance against micromelia and beak defects, again without regard to the chemical constitution of the teratogenic compound. The nature of these differences will be further discussed below. Secondly, altered biochemical situations are evident from the toxicity relationships of various supplements. It has been shown, for instance, that pyruvic acid, while reducing insulin toxicity (as well as its teratogenic effects) during the rumplessness-inducing stages and being quite harmless when given alone, becomes extremely toxic (and loses its protective properties) probably prior to the establishment of circulation and certainly by the time when insulin and other chemicals lead to micromelia. Similar situations exist for lactic, citric and l-glutamic acid. Conversely, substances such as nicotinamide and glucose-1-phosphate, are definitely toxic during the early embryonic stages when they have little or no protective value as far as the origin of rumplessness goes, but are non-toxic later when they serve to reduce or prevent the production of micromelia and/or beak abnormalities. All these statements can, of course, be only made with assurance for the dosages used in our experiments. Still other evidence of a similar kind comes from the distinctive manner in which various substances potentiate the teratogenic insulin effects at one stage, but not at another. Thus adrenal cortex extract has no influence on the manner of occurrence of rumplessness after insulin, but forcefully potentiates incidence and ex-

pression of the abnormalities induced by insulin during the 4th or 5th day (Landauer, '47a); contrariwise, phosphopyruvic acid (2 mg/egg) and glutamine (1.8 mg/egg) greatly exaggerate the effects of insulin during the rumplessness-inducing period (unpublished data), but do not produce similar results when added to insulin during the micromelia-inducing stages. All these observations point to the existence of important physiological dissimilarities in the origin of abnormalities during the two developmental periods.

Even at one and the same stage one finds response differences to supplementation which any attempt at interpretation must take into account. Insulin treatment at 96 or 120 hours produces micromelia, beak defects and syndactylism, the incidence of the three types of defects decreasing in the given order. These variations in frequency of occurrence may be determined by developmental differences between the several primordia at the time of treatment or by dissimilarities in their quantitative dependence on the processes that are disturbed by insulin treatment. There are indications, however, that even in these situations qualitative variations in local functions may play an important role. This is suggested by our evidence for differential protection and differential potentiation following treatment with insulin and various supplements. Instances of differential protection (in experiments at 96 hours of incubation) include our observation that lactic acid supplementation reduced the incidence of insulin-induced micromelia, but not that of beak defects, or, that citric acid, glucose-1-phosphate and yet other substances protect against insulin-beak defects, but not against micromelia. Differential potentiation is well illustrated by the fact that supplementation of insulin treatment at 96 hours with 3-hydroxy-anthranilic acid produced relatively the greater potentiation the lower was the incidence of a particular abnormality after insulin alone (order of potentiation: syndactylism > beak defects > micromelia). Sodium succinate supplementation gave similar results. It would be difficult to interpret these observations in any fashion other than the occurrence of dif-

fundamental biochemical effects of insulin treatment on various organs and parts. It is quite possible that the primary action of insulin is the same in all instances, but that the results flowing from it vary according to the metabolic importance of the affected substance at the time of treatment and that the effectiveness or toxicity of supplements is in part determined by changing substrate requirements.

The extreme complexity of the teratogenic mechanisms that are responsible for our results is sufficiently clear. At the present stage of almost complete ignorance about the biochemical processes involved in morphogenesis and differentiation one cannot expect to define more than the most general outlines of the events that took place under the conditions of our experiments. It cannot be doubted, at any rate, that the principal, if not only, primary disturbance which, in our material, leads to abnormal development is an interference with embryonic carbohydrate metabolism. This is evident from the nature of the teratogenic substances, from the type of compounds which have preventative effects when used as supplements, and from the kinds of substances acting as potentiators of the teratogenic agents. Additional evidence from experiments with inhibitors of glycolysis will be presented below. This general conclusion is, of course, in good agreement with what is known about the importance of carbohydrate utilization in early development.

Our results also demonstrate clearly, however, that between our "early" (0 or 24 hours) and "late" (96 or 120 hours) stages of experimentation important differences exist in the manner in which the embryonic carbohydrate metabolism is interfered with by our procedures. Before proceeding with a discussion of these differences, it will be well to present the results of experiments with various inhibitors of anaerobic glycolysis. We tested the effect of sodium malonate, sodium fluoride, glyceraldehyde, phlorizin and monoiodoacetic acid on development of the chicken embryo at 0, 30 and 96 hours. The results are shown in table 13. This table indicates only the incidence of rumplessness at 0 and 30 hours and of

micromelia and beak abnormalities at 96 hours; no other defects occurred in significant numbers. During the two early stages phlorizin and monoiodoacetic acid produced rumplessness in highly significant frequencies; both were more effective at 30 hours than at 0 hours. Among a large number of tested substances, monoiodoacetic acid ranks with insulin and Ancel's arsenic compounds (sodium cacodylate and so-

TABLE 13

The effects of inhibitors of anaerobic glycolysis at 0, 30 and 96 hours of development. White Leghorn embryos

TREATMENT	AT 0 HOURS		AT 30 HOURS		AT 96 HOURS	
	Number	Rumplessness	Number	Rumplessness	Number	Micromelia and beak defects
		%		%		%
Sodium malonate (10 mg/egg)	57	3.5	[1]	[1]	129	0.8
Sodium fluoride (40 γ/egg)	101	0	131	3.8	152	0
Glyceraldehyde (63 γ/egg)	101	4.0	134	3.0	146	0
Phlorizin (250 γ/egg)	146	0.9	139	8.6	148	0
Monoiodoacetic acid (0.6 mg/egg)	96	6.3	83	17.2	120	0

[1] Not tested.

dium methyl arsonate) as one of the most potent agents for the production of rumplessness. None of the 5 inhibitors had significant teratogenic effects at 96 hours; this was even true when twice the amounts indicated in the table were used and in spite of the fact that some of the inhibitors, e.g. monoiodoacetic acid, were definitely toxic.

In view of these observations as well as of the evidence presented previously there can be little doubt that in our experiments the production of rumplessness between 0 and

30 hours of incubation had its origin in a disruption of anaerobic glycolysis. It is more hazardous to speculate on the point of interference within the metabolic chain, but some interesting facts should be pointed out. To start with, it is known that the pyruvate oxidase system (pyruvate dehydrogenase) is especially sensitive to arsenical poisoning. This has been shown for trivalent organic arsenicals (Barron and Singer, '49) and for arsenite (Sinclair, quoted from Bernheim, '48; Cohen-Bazire et al., '48), and our evidence (Landauer, '49) suggests that the same holds for *organic* arsenic compounds of pentavalent origin. On the assumption that the arsenic compounds used by Ancel and ourselves interfere with steps between phosphopyruvate and lactate and thereby produce rumplessness, it would become understandable that pyruvate and lactate are effective in preventing or reducing the occurrence of rumplessness and that phosphopyruvic acid, given in addition to one of the teratogenic agents (insulin), not only is very toxic, but greatly increases the incidence of rumplessness.[5] It seems reasonable to assume that insulin treatment blocks the coenzyme supply and that pyruvic acid during these early and predominantly anaerobic stages of development aids in the reoxidation of the pyridine nucleotides, thereby making them again available to the embryo. The fact that monoiodoacetic acid, among the inhibitors tested, stands out for its effectiveness in producing rumplessness also fits well into this interpretation. Finally, Spratt ('50) has shown in explantation experiments that the damaging effects of monoiodoacetic acid can be prevented by substituting pyruvate or lactate for the glucose of the culture medium. It must be admitted, however, that many of the facts relating to the experimental production of rumplessness could also be explained on the basis of interference with phosphorylating mechanisms. No final answer can at present be given.

[5] The combination of two units insulin and 2 mg phosphopyruvic acid injected at 24 hours produced a mortality of 83.5% during the first 6 days. Among the survivors of the 17th day 42% were rumpless. At 96 hours phosphopyruvic acid did not have similarly toxic or potentiating effects. Reduced glutathione did not protect against sodium cacodylate-induced rumplessness.

The stages during which rumplessness is most readily produced by teratogenic substances occur prior to the existence of circulation, and the action of these substances is presumably directly on cellular functions of the embryo itself. At the later period, however, when the embryo is likely to respond with micromelia and beak abnormalities, the circulatory system is well established. It is true that even at this stage the endocrine regulation of carbohydrate utilization has not yet come into being, the embryos in this respect still resembling the hypophysectomized and pancreatectomized adult mammal, but the yolk sac membrane has begun to function as "transitory liver." The investigations of Zwilling ('51b) have shown that one of the agents inducing micromelia and beak abnormalities, viz. insulin, has profound effects on the carbohydrate storage functions of the yolk sac membrane, thereby producing hypoglycemia and carbohydrate starvation of the embryo (Zwilling, '48, '51a b). Whether or not the disturbances of yolk sac membrane functions are the only primary consequence of insulin treatment at this stage, carbohydrate deficiency and malformations of the embryos being a consequence, remains to be determined. It seems at present more likely that the teratogenic agents responsible for micromelia and beak abnormalities have direct, as well as indirect, effects on the embryo since a similar syndrome of defects (and one also preventable by nicotinamide supplementation) follows sulfanilamide treatment in the absence of hypoglycemia (Zwilling and DeBell, '50).[4] Moreover, the same syndrome or similar ones can be produced by treatment with eserine sulfate or by deficiencies of the maternal diet in biotin, riboflavin and manganese, none of which conditions are known or likely to be accompanied by changes in the blood sugar level.

Taking into account the great stage-dependent differences in effect which supplementary substances, such as nicotinamide and pyruvic acid, produce in conjunction with insulin,

[4] It is interesting in this connection that according to Mendes Alves ('46) sulfanilamide and other sulfa compounds reduce the hypoglycemic effects of insulin and that this anti-insulin action is removed by nicotinamide.

and in view of the fact that inhibitors of anaerobic glycolysis which are teratogenic in early development become non-teratogenic later on, it must be concluded that during the transition from the 24-hour stage to that at 96 hours important changes have occurred in the normal metabolic activities of chicken embryos. It may be suspected that the altered responses are related to the increasing importance of aerobic respiration, as indicated *inter alia* by the sudden rise in cytochrome oxidase after the third day of development (Albaum and Worley, '42; Albaum, Novikoff and Ogur, '46). Establishment or increasing importance of the tricarboxylic acid cycle may be a factor.

In view of the high efficiency with which nicotinamide protects between 96 and 120 hours of embryonic development, against the teratogenic effects of insulin, there can be little doubt that during this period insulin interfered with coenzyme dehydrogenase activity.[5] This is, in any event, the only established function of nicotinamide (via the pyridine nucleotides). It seems likely that, as in the earlier rumplessness-producing stages, insulin temporarily reduced or abolished existing coenzyme supplies, possibly by competing for the apoenzyme, but that at this later stage with its different respiratory activities nicotinamide (rather than pyruvic acid) is needed as a supplement in order to insure the availability of adequate amounts of coenzyme. Most of our observations with various supplements, whether beneficial or harmful, can be reconciled with this view. In particular does it become understandable that nicotinamide, in excessive amounts (equal amounts being more excessive in early stages), is likely to be toxic and teratogenic by acting as an inhibitor of its own coenzyme.

Certain pathways which might be suspected to play a role in the origin of our malformations are ruled out by available evidence. Inhibition of succinic dehydrogenase activity seems excluded since several inhibitors of this system (sodium

[5] Measurements are in progress.

malonate, triphenyl tetrazolium chloride and oxaloacetic acid) have no teratogenic effects on 96-hour chicken embryos, even when given in toxic amounts.

Since eserine is one of the substances producing micromelia and beak defects, it might be asked if the origin of these abnormalities is related to anticholinergic action. This is very unlikely for two reasons. The anticholinergic consequences of eserine presumably occur after much smaller amounts of the drug than Ancel and ourselves found effective for the production of abnormalities. Furthermore, unpublished experiments with diisopropylfluorophosphate (0.2 cm³/egg of 0.1% solution) at 96 hours gave no indication that this anticholinergic substance is teratogenic. It has been shown, on the other hand, that eserine sulfate, in such concentrations as are needed for the production of micromelia, leads to interference with processes of dehydrogenation (Brooks, Rensmeier and Gerard, '49), and it is in better accord with all our other evidence to assume that a mechanism of this kind is responsible for the micromelia-inducing action of eserine.

It is certain that the ultimate results of those biochemical changes which are impressed upon embryos by teratogenic substances may be modified in various ways by conditions existing in the embryos themselves, but we have little specific information about the manner in which these modifications are produced. One such modifying agency which has been noted again and again in our work is related to the age of the pullets from which eggs had been obtained. It finds expression in a gradual decline in response of embryos to insulin from early spring to mid-summer. This has been found true with regard to the incidence of rumplessness as well as of micromelia and beak defects. It seems likely that this change of response is produced by a regular trend of some kind in the composition of eggs, such as has been recorded by Osonka ('50) for nitrogen, methionine and cystine content.

Another source of variation in response to teratogenic substances, relating more to manner and degree of expression

than to incidence, is probably to be found in the exact developmental (and therefore presumably biochemical) condition of a particular organ primordium at the moment at which a teratogenic substance takes effect. Such relatively minor differences in developmental age presumably decide in which of several alternative morphological patterns rumplessness arises after insulin treatment (Moseley, '47) or in which of several possible directions beak development deviates from the norm. The lesser effectiveness of nicotinamide in preventing insulin-induced changes of expression of polydactylism as compared with its protective value against micromelia may be related to the nearness of irreversible determination of the toe primordia at the time of treatment. However, at this same time, i.e. 96 hours of development, it is still possible to produce in normal-toed stock a differential rise in the incidence of insulin-induced syndactylism by giving 3-hydroxy-anthranilic acid in addition to insulin. The developmental origin of syndactylism presumably occurs very soon after that of polydactylism. It seems, therefore, that for changes in the pattern of toe development the time of maximum sensitivity falls near the moment of irreversible differentiation.

A different situation obtains in regard to growing bones. Insulin treatment produces micromelia and, if less frequently, beak defects of chicken embryos as late as 168 hours of development (Landauer, '47) and probably still later. The long duration of susceptibility of the growing long bones to insulin is presumably accounted for by the special importance of glycogen storage in cartilage (for review see Follis and Barthrong, '49) and the existence of a phosphorylating system associating glycogenolysis with calcification (Gutman and Gutman, '41; Gutman, Warrick and Gutman, '42). Reduced glycogen stores and/or impaired glycogenolytic activity probably slow down calcification and, indirectly, many other processes of growing bones. Interference of this kind can be expected from the time calcification begins (or perhaps even earlier) to the end of cartilage replacement by bone. Hence

the protracted effectiveness of insulin in calling forth micromelia and allied defects. It remains to be explained, however, what causes the differential response of various skeletal parts, i.e. why, irrespective of the time of treatment (and also in all the mutant forms), the long bones of the leg are more affected by abnormal conditions than are those of the wing.

Many other details of our observations must remain unexplained for the time being. It is not clear, for instance, why l-glutamic acid hydrochloride affords a significant, if low, degree of protection against rumplessness after insulin-treatment, but tends to produce a certain incidence of rumplessness, when given by itself.

It is a common experience that substances of very different chemical constitution have similar pharmacological effects, but only in exceptional cases has their common pathway been discovered. This has often led students to the premature conclusion that common pathways do not exist. In the analysis of biological problems such situations have frequently been disposed of with the inference that particular responses are of an "unspecific" nature, a concept to which many shades of meaning became attached.

The idea that an "unspecific" retardation or arrest of development is the cause of malformations has a long history in which appear the names of Harvey, Haller, C. F. Wolf, Etienne and Geoffroy Saint-Hilaire, and Dareste. Its best known modern proponent, C. R. Stockard ('21) put it in the following words: "First, all types of monsters, double as well as single, may be caused by one and the same treatment; second, any one type of monster . . . may be produced by a great number of different experimental treatments; third, all effective treatments tend primarily to lower the rate of development, and, fourth, the type of monster induced depends upon the particular developmental moment or moments during which the developmental rate was reduced."

The work of Ancel and our own experiments have shown conclusively that treatment of chicken embryos at one and the same developmental stage will lead to different results

depending on the particular chemical agent to which the embryo was exposed, e.g. sodium cacodylate will give rise to rumplessness in 24-hour embryos, but not to micromelia at 96 hours, eserine sulfate or sulfanilamide will induce micromelia at 96 hours, but not rumplessness at 24 hours. It is a fact that one and the same type of abnormality frequently can be produced by several, and often chemically unrelated, substances, but wherever tests with supplementation have been applied, it could be shown that specific derangements of metabolism caused particular defects. This, of course, does not preclude the possibility that one and the same malformation may result from more than one type of biochemical interference. Nor is it justifiable to point, in support of Stockard's generalization, to our observation that one and the same compound, e.g. insulin, may produce several types of malformations depending on the developmental stage of treatment. For, our experiments with metabolic supplements make it clear that this versatility of insulin is due to a multiplicity of reactions rather than a lack of specificity.

If Stockard's third principle was meant to imply that *any* lowering of developmental rate at a susceptible period will *ipso facto* be responsible for the subsequent occurrence of malformations, it is demonstrably untrue as is shown by many cooling experiments with developing chicken embryos. But even where, following treatment with teratogenic substances, the emergence of morphological defects is associated with growth retardation, a causal relationship should not be assumed without proof. In the case of sulfanilamide, for instance, Zwilling and DeBell ('50) have shown that growth retardation and micromelia are independent and separable effects.

It is true that many of the teratogenic substances used by us are known to have inhibitory effects on mitosis. This has been shown, e.g., for insulin (Bullough, '49; Bullough and Elsa, '50), sulfanilamide (Fisher, Henry and Low, '44), arsenic compounds (Ting and Ludford, '50) and monoiodoace-

tic acid. Not only was it possible, however, to separate, in the case of sulfanilamide, growth retardation and teratogenesis, but many of our malformed embryos and chicks had normal body weight. Since the publication of Stockard's paper the origin of many abnormalities has been explained on the basis of interference with mitotic activity and embryo growth (e.g. Bullough and Abdel-Malek, '49), but in many of these instances closer scrutiny may yet trace the causal sequence to metabolic upsets within particular primordia.

Finally, the 4th of Stockard's principles can only be accepted with reservations. We could demonstrate, for instance, that the latitude of susceptible periods, in regard to a certain defect, varies with the dose of the particular teratogenic substance or that for different teratogenic agents the maximum period of susceptibility differs with respect to one and the same abnormality.

All our observations point to the conclusion that, as development proceeds, there are shifting substrate requirements and changing enzyme activities in different parts of the embryo and that the resulting internal competition is responsible for the occurrence of critical periods and their attendant dangers.

The functioning of particular enzyme systems and the availability of and needs for particular substrates presumably are inextricably interwoven during development and dependent on each other. But the immediate cause of the failure of certain organ primordia to develop normally after treatment with teratogenic agents must be looked for in a lack or insufficient supply of necessary substrate and it may be concluded from our observations that selective starvation plays an important role in the origin of malformations. The pattern of abnormal development varies with the stage attained at the time of treatment, but differs considerably from that observed on explanted blastoderms (Spratt, '50). The selective susceptibility of skeleton-forming material is one of the outstanding features of our observations.

SUMMARY

Our observations can be summarized as follows.

With proper dosage and timing supplementary nicotinamide protected against the abnormalities (micromelia and beak defects) produced by insulin-injection at 96 or 120 hours of development.

Nicotinamide decreased to some extent the modifying action of insulin on the expression of hereditary polydactylism.

The incidence of rumplessness after insulin at 24 hours was only slightly reduced by supplementary nicotinamide.

At 24 hours, but not at 96 or 120 hours, embryo mortality after insulin-treatment was much increased by supplementary nicotinamide. At the earlier developmental stage nicotinamide had considerable toxicity when given alone and was responsible for the occurrence of beak abnormalities.

The incidence of beak defects after insulin at 96 hours could be selectively reduced by supplementary glucose-1-phosphate, citric acid, oxalacetic acid and, probably, fructose-6-phosphate. There was no similar lowering in abnormalities of the extremities (micromelia, syndactylism).

Pyruvic acid, when given as a supplement to insulin-treatment at 24 hours, greatly reduced the incidence of rumplessness and lowered embryo mortality. The same was not true for phosphopyruvic acid. Results similar to those with pyruvic-acid supplementation, if less spectacular, followed supplementation with lactic and citric acid. Isocitric acid was highly toxic and increased the incidence of rumplessness.

In later developmental stages pyruvic acid was very toxic and in combination with insulin potentiated the teratogenic effects of the latter. Lactic acid also became toxic at later stages, but, when given three hours prior to insulin, it still had some protective value.

The l-glutamic acid hydrochloride, when injected by itself at 24 hours, had a low rumplessness-inducing effect, but it reduced the incidence of rumplessness after insulin treatment when used as a supplement.

Sodium succinate and 3-hydroxy-anthranilic acid potentiated the effect of insulin at 96 hours. This potentiation was the greater the lower was the incidence of a particular abnormality after insulin alone. Given by themselves both compounds were harmless.

Malformations produced by insulin-treatment at 96 or 120 hours, and not heretofore reported, are syndactylism and cleft palate, the latter associated with coloboma of the face.

Tests with inhibitors of anaerobic glycolysis (sodium malonate, sodium fluoride, glyceraldehyde, phlorizin, monoiodoacetic acid) showed that phlorizin and especially monoiodoacetic acid, injected at 24 hours of incubation, produced a considerable incidence of rumplessness. None of these inhibitors were teratogenic at 96 hours.

Our results demonstrate that certain substances provide protection against those teratogenic compounds which in 24-hour embryos tend to induce rumplessness, that other substances forestall, completely or in part, the production by chemical interference at the 96- or 120-hour stage of micromelia, syndactylism and beak defects, that some supplements furnish differential protection (e.g. against beak defects, but not against micromelia), and that other supplements produce differential potentiation of the insulin effects (e.g. raising the incidence of syndactylism much more than that of beak abnormalities). It follows that the metabolic situations upon which act the teratogenic agents as well as the substances used as supplements, vary not only between different developmental stages, but also between different organ primordia of one and the same stage.

It seems likely that the teratogenic effects of insulin are produced by rendering coenzyme unavailable to the embryo. During the early and predominantly anaerobic stages of development pyruvic acid, by aiding reoxidation of the pyridine nucleotides, presumably serves to prevent insulin damage. During later stages, and on account of the changed respiratory conditions of the embryo, nicotinamide supplementation is required to offset the blocking effect of insulin on the co-

enzymes. Chemicals which resemble insulin in their teratogenic effects probably do so by interference with the same or closely related metabolic steps. Supplements to insulin treatment seem to be beneficial or noxious according to whether they aid or hinder in relieving the insulin determined scarcity of coenzyme.

The long duration of susceptibility to insulin treatment of the long bones and other skeletal parts can be explained on the basis of the special significance of glycogenolysis in bone calcification.

The principles of "unspecific" retardation of embryo growth, as enunciated by Stockard and others, cannot explain the origin of the teratogenic processes here described.

ACKNOWLEDGMENTS

This work was done under a grant from the American Cancer Society, on recommendation of the Committee on Growth of the National Research Council. Financial support was also received from the International Baby Chick Association.

The Retin (Insulin, Lilly) was generously provided by the Lilly Research Laboratories through the courtesy of Dr. F. B. Peck.

We are under special obligation to Drs. Heinrich Waelsch and Heinz Herrmann for valuable advice.

LITERATURE CITED

ALBATH, H. G., A. B. NOVIKOFF AND M. OKUN 1946 The development of the cytochrome oxidase and succinoxidase systems in the chick embryo. J. Biol. Chem., *165*: 125–132.

ALBATH, H. G., AND L. G. WORLEY 1942 The development of cytochrome oxidase in the chick embryo. J. Biol. Chem., *144*: 697–702.

ANCEL, P. 1945a L'achondroplasie. Sa reduction expérimentale, sa pathogénie. Ann. d'Endocrinol., *6*: 1–44.

——— 1945b Les variations individuelles dans les expériences de tératogenèse. La Revue Scient., *83*: 80–102.

——— 1950 La chimiotératogenèse chez les vertébrés. Paris (G. Doin and Cie).

BARRON, E. S. G., AND T. P. SINGER 1942 Enzyme systems containing active sulfhydryl groups. The role of glutathione. Science, *97*: 239–240.

BERNHEIM, F. 1948 The Interaction of Drugs and Cell Catalysis. 2nd edition. Minneapolis (Burgess).

BLUTTNER, R. J., AND A. P. WILLIAMSON 1950 Abnormalities in the beaks of developing chick embryos. Postmortem changes which may be confused with developmental anomalies. Archives of Pathology, *50*: 670–675.

BONNSTEIN, D., AND A. ANDRE-MALEK 1949 The induction of eurtapedia by nitrogen mustard in Drosophila virilis. J. Exp. Zool., *111*: 97–115.

BULLOUGH, W. S. 1949 Epidermal mitosis in relation to sugar and phosphate. Nature, *163*: 676–677.

BULLOUGH, W. S., AND E. A. DIXA 1950 The diurnal variations in the tissue glycogen content and their relation to mitotic activity in the adult male mouse. J. Exp. Biol., *27*: 257–[illegible].

BROOKS, V. B., R. E. HANSHEIM AND R. W. GERARD 1949 Action of anticholinesterases, drugs and intermediates on respiration and electrical activity of the isolated frog brain. Am. J. Physiol., *157*: 290–310.

COHEN-BAZIRE, G., G. N. COHEN, B. NICHAS AND M. RAYNAUD 1948 Action inhibitrice de l'arsénite de sodium sur la production d'acide butyrique à partir de pyruvate, chez Cl. saccharobutyricum. C. R. Société de Biologie, *142*: 1999–2000.

CSONKA, F. A. 1950 Nitrogen, methionine and cystine content of hen's eggs. Their distribution in the egg white and yolk. J. Nutrition, *42*: 445–451.

DANFORTH, C. H. 1919 The developmental relations of brachydactyly in the domestic fowl. Am. J. Anat., *25*: 97–115.

EVANS, H. M., M. M. NELSON AND C. W. ASLING 1951 Multiple congenital abnormalities resulting from acute folic acid deficiency during gestation. (Abstract.) Science, *114*: 473.

FISHER, K. C., R. J. HEINT AND E. LOW 1944 The effects of sulfanilamide and azide on oxygen consumption and cell division in the egg of the sea urchin, Arbacia punctulata. J. of Pharmacology and Experimental Therapeutics, *81*: 58–63.

FOLLIS, R. H., JR., AND M. BRUNSTRONG 1948 Histochemical studies on cartilage and bone. I. The normal pattern. Bulletin of the Johns Hopkins Hospital, *83*: 251–267.

GILLMAN, J., C. GILBERT AND T. GILLMAN 1949 A preliminary report on hydrocephalus, spina bifida and other congenital anomalies in the rat produced by trypan blue. South African J. of Med., *23*: 47–60.

GUTMAN, A. B., AND E. B. GUTMAN 1941 A phosphorylase in calcifying cartilage. Proceedings of the Society for Experimental Biology and Medicine, *48*: 627–631.

GUTMAN, A. B., F. B. WAELSCH AND E. B. GUTMAN 1942 Phosphorylative glycogenolysis and calcification in cartilage. Science, *95*: 461–462.

HAGALLS, T. H., F. J. CURLEY AND R. A. PETERS 1950 Anoxia as a cause of fetal death and congenital defect in the mouse. Am. J. of Diseases of Children, *80*: 34–42.

HAAP, R. G. 1949 Brachydactyly and syndactyly in ptilopod domestic fowl. Proc. Oklahoma Acad. Sci., *29*: 27–32.

KING, H., AND R. J. LUDFORD 1950 The relation between the constitution of arsenicals and their action on cell division. J. Chem. Soc. (London), pp. 2086–2088.

LANDAUER, W. 1945 Rumplessness of chicken embryos produced by the injection of insulin and other chemicals. J. Exp. Zool., *98:* 65–77.

——— 1947a Potentiating effects of adrenal cortical extract on insulin-induced abnormalities of chick development. Endocrinology, *41:* 489–493.

——— 1947b Insulin-induced abnormalities of beak, extremities and eyes in chickens. J. Exp. Zool., *105:* 145–172.

——— 1948a Hereditary abnormalities and their chemically-induced phenocopies. Growth Symposium, *13:* 171–200.

——— 1948b The effect of nicotinamide and α-ketoglutaric acid on the teratogenic action of insulin. J. Exp. Zool., *109:* 283–290.

——— 1948c The phenotypic modification of hereditary polydactylism of fowl by selection and by insulin. Genetics, *33:* 133–157.

——— 1949 Le problème de l'électivité dans les expériences de tératogenèse biochimique. Archives d'Anatomie microscopique et de Morphologie expérimentale, *38:* 184–189.

LANDAUER, W., AND C. I. BLISS 1946 Insulin-induced rumplessness of chickens. III. The relationship of dosage and of developmental stage at time of injection to response. J. Exp. Zool., *102:* 1–22.

MENDES ALVES, M. A. 1946 Acções organotrópicas das sulfonamidas. Diss. Lisboa.

MOSELEY, H. R. 1947 Insulin-induced rumplessness of chickens. J. Exp. Zool., *105:* 279–316.

SPRATT, N. T., JR. 1950 Nutritional requirements of the early chick embryo. III. The metabolic basis of morphogenesis and differentiation as revealed by the use of inhibitors. Biological Bulletin, *99:* 120–135.

STOCKARD, C. R. 1921 Developmental rate and structural expression: an experimental study of twins, "double monsters" and single deformities, and the interaction among embryonic organs during their origin and development. Am. J. Anat., *28:* 115–266.

WARREN, D. C. 1950 Syndactylism in the chicken. J. of Heredity, *41:* 31–34.

WARKANY, J., R. C. NELSON AND E. SCHAFFENBERGER 1943 Congenital malformations induced in rats by maternal nutritional deficiency. IV. Cleft palate. Am. J. of Diseases of Children, *65:* 882–894.

ZWILLING, E. 1948 Association of hypoglycemia and insulin micromelia in chick embryos. J. Exp. Zool., *109:* 197–214.

——— 1951a Insulin-induced hypoglycemia and rumplessness in chick embryos. J. Exp. Zool., *117:* 65–73.

——— 1951b Carbohydrate metabolism in insulin-treated chick embryos. Archives of Biochemistry and Biophysics, *33:* 228–242.

ZWILLING, E., AND J. T. DEBELL 1950 Micromelia and growth retardation as independent effects of sulfanilamide in chick embryos. J. Exp. Zool., *115:* 59–81.

455

15264

Effect of Acetic, Monochloroacetic, Dichloroacetic, and Trichloroacetic Acids on Oxygen Consumption of Mouse Liver.

EDWIN P. LANG. (Introduced by O. Garth Fitzhugh.)

From the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

Proc. Soc. Exp. Biol. and Med. 61(2): 178-179, (1946)

From acute oral toxicity data in mice, rats and guinea pigs[1] it has been shown that monochloroacetic acid is from 25 to 40 times as toxic as acetic acid and its di- and trichloro derivatives. It has not been determined from these studies what causes death nor have chronic feeding experiments indicated any clearly defined functional or organic derangements. While the effect of monoiodoacetic acid on the glycolytic and oxidative functions of tissues has been amply demonstrated, such information on monochloroacetic acid is at best somewhat meager. It has been recorded that monochloroacetic acid has cholagogic action[2] and Lundsgaard[3] states that in comparison to monoiodoacetic acid, monochloroacetic acid inhibits yeast fermentation only feebly. The present experiments were undertaken to determine what effect, if any, mono-, di- and trichloroacetic acid would have on the respiration of liver tissue.

Method. Liver slices from well fed young adult mice were used. Oxygen consumption was measured by the standard Warburg procedure at 37.5 C. Acetic, mono-, di-, and trichloroacetic acids were neutralized and diluted in phosphate buffered glucose-Ringer, so that the final concentration in contact with the liver slice ranged from 0.05M to 0.00005M. The pH of all solutions was adjusted to 7.4.

Results. Fig. 1 shows in graphic form the typical effects of mono-, di-, and trichloroacetic acids at 0.0005M concentration on the respiration of mouse liver slices. Respiration is promptly and precipitously reduced by

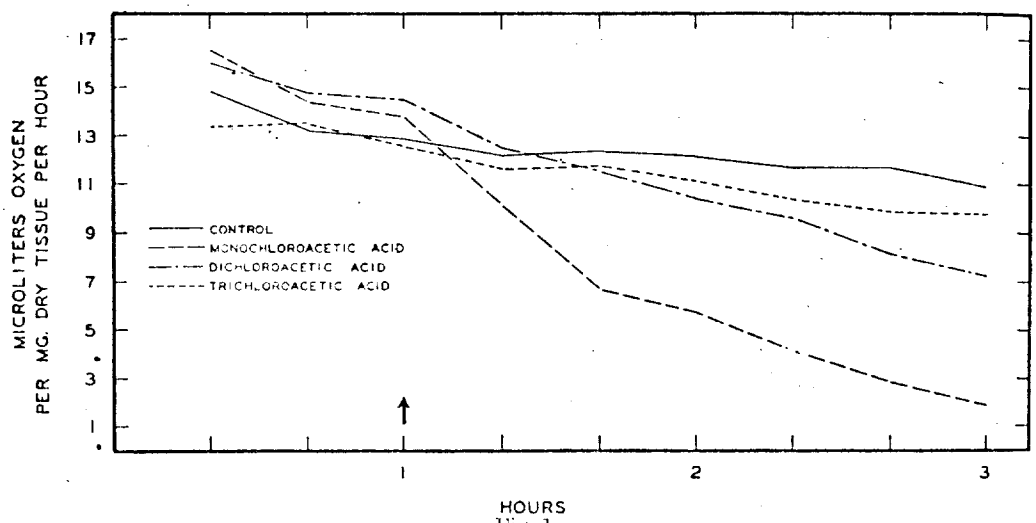


Fig. 1.

Effect of 0.005M mono-, di-, and trichloroacetic acids on oxygen consumption of mouse liver slices. Temp. 37.5° C. pH 7.4.

[1] Woodard, G., Lange, S. W., Nelson, K. W., and Calvery, H. O., *J. Indust. Hyg. and Tox.*, 1941, **23**, 78.

[2] Chabrol, E., Charonnat, R., Maximin, M., and Waitz, R., *Compt. rend. Soc. Biol.*, 1931, **106**, 17.

[3] Lundsgaard, E., *Biochem. Z.*, 1932, **250**, 61.

monochloroacetic acid, and within 2 hours almost completely abolished. The effect of di- and trichloroacetic acids, while clearly defined, is much weaker and more delayed. It is important to note that here, as at all other concentrations studied, the order of decreasing effect on respiration is: monochloroacetic acid > dichloroacetic acid > trichloroacetic acid. Acetic acid in the range of 0.01M to 0.001M showed no effect.

Within the range of 0.001M to 0.03M, the depression of respiration by mono- and dichloroacetic acids is maximal; there is no indication of a progressively increasing effect with increasing concentration. Within the range of 0.001M to 0.00005M there is a gradually decreasing effect, which ceases below 0.00005M for both acids. This is a concentration of the order of 5 to 10 μg per milliliter of Ringer solution. At a concentration below 0.005M no effect of trichloroacetic acid has been noted, and at 0.01M evidences of tissue coagulation have been seen.

Summary. At the concentrations studied monochloroacetic acid produces a marked depression in the respiration of mouse liver slices. The order of decreasing effect of the 3 acids is: monochloroacetic > dichloroacetic > trichloroacetic. Acetic acid has no effect.

15265

Electrophoretic Analysis of Kala-azar Human Serum. Hypergammaglobulinemia Associated with Seronegative Reactions for Syphilis.*

G. R. COOPER, CHARLES R. REIN, AND J. W. BEARD.

From the Department of Surgery, Duke University School of Medicine, Durham, N. C., and the Division of Serology, Army Medical School, Army Medical Center, Washington, D. C.

It is generally recognized that nonspecific positive reactions in tests for syphilis may occur with human sera from normal individuals[1,2] and from individuals affected with certain diseases[3-5] and conditions not associated with syphilis. The belief has arisen among serologists that nonspecific positive reactions are associated with hyperglobulinemia, and the presence of a high serum protein or globulin content has been suggested[6] as a criterion of the nonspecificity of a positive reaction.

The disease, kala-azar, is characterized in part by hyperglobulinemia[7] associated with the presence in high concentration of abnormal euglobulin, which gives rise to the formol-gel[8] reaction and a cold-precipitable pathological globulin.[9] In this disease, also, there has been reported[10] the occurrence of nonspecific positive reaction in tests for syphilis. Recently, in connection with a comparative study

* The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Duke University.

1 Eagle, H., *Am. J. Syph.*, 1941, **25**, 7.

2 Mohr, C. F., Moore, J. E., and Eagle, H., *Arch. Int. Med.*, 1941, **68**, 808.

3 Mohr, C. F., Moore, J. E., and Eagle, H., *Arch. Int. Med.*, 1941, **68**, 1161.

4 Hazen, B. H., Pettit, T., Sanford, A. H., Sweat, F. E., Simpson, W. M., and Vonderlehr, R. A., *Internat. J. Leprosy*, 1936, **4**, 315.

5 Davis, B. D., *Medicine*, 1944, **23**, 559.

6 Cardon, L., and Arias, D. H., with the assistance of Arias, E., Brancos, M. J., Tolchinsky, S. L., and Brancos, J., *Arch. Dermat. and Syph.*, 1942, **46**, 713.

7 Lloyd, R. B., and Paul, S. N., *Indian J. Med. Res.*, 1928-1929, **16**, 203.

8 Napier, L. E., *Indian J. Med. Res.*, 1921-1922, **9**, 804.

9 Wormser, E., and Stolz, L., *J. Lab. and Clin. Med.*, 1944, **29**, 1082.

10 Chowdhury, S. D., Sengupta, P. C., and Napier, L. E., *Indian J. Med. Res.*, 1929-1949, **27**, 181.

TREATMENT OF TRACHOMA BY ACETIC ACID

Brit. J. Ophth 11; 150-152

BY
J. A. LEA
NATAL

HAVING seen lately in the BRITISH JOURNAL OF OPHTHALMOLOGY several papers, more especially abstracts from foreign sources on the treatment of trachoma, and having noticed how varied the treatment was, and in some instances severe, I am encouraged to give my experience of a plan of treatment which I have found successful during the time that I have practised as an ophthalmic surgeon in South Africa.

When first I came to South Africa several years ago I found trachoma very prevalent, more especially in the dry, dusty up-country districts; and had many severe and neglected cases to attend. I used the usual remedies with only moderate success. I then commenced this plan of treatment which I followed with success until I retired from practice a few years ago.

My method of procedure is as follows: I wash the eye and eyelids with a saturated solution of boracic acid. I then drop in a 0.5 per cent. solution of cocaine (I use Darier's solution which contains also adrenaline). I then take an instrument made for

me by Messrs. Weiss (an ordinary tattooing needle will do), dip it into a mixture of equal parts of acetic acid and water taking care to fill the groove, and having everted the eyelid, prick every true granulation and also very thoroughly any wavy rolls, or fringes of conjunctiva; wash again with a solution of boracic acid; and apply a cold compress to the eyelids for an hour or so.

This can be repeated two or even three times a week, and when the true granulations have disappeared I apply sulphate of copper to the hypertrophied papillae, alternating this with a 2 per cent. solution of nitrate of silver; more especially if the sulphate of copper causes too much irritation.

I have been astonished at the rapidity with which the true granulations, and wavy appearance of the conjunctiva disappeared after pricking with the acetic acid.

It is easy to do, as the true granulations are soft, the needle sinks in by its own weight, and consequently the operation is practically painless if carefully done.

It is useless as an application to the false granulations for which sulphate of copper seems to be a specific. I like to use a fine-pointed stick of sulphate of copper, and apply carefully to the granulations washing off any excess with a solution of boracic acid.

I have noticed in reading papers on the subject of trachoma that there is not only a want of unanimity in the methods of treatment, but there is also some want of agreement as to what constitutes a case of trachoma. I will therefore describe the kind of case seen in South Africa.

On everting the eyelids a velvety red surface interspersed with grey translucent dots is seen: these dots vary in size from that of a pin's head to that of a hemp seed; and in some cases in the fornices there are rolls of wavy-looking tissue; there is frequently some amount of pannus, and sometimes a facetted ulcer, or ulcers on the cornea. The amount of discomfort is usually less than one would expect from the appearance of the eye; and the ulcers have little tendency to break down or slough. These true granulations are soft, as are also the wavy masses, differing from the hypertrophied papillae which are firm, and the granulations of spring catarrh which are very hard. One often sees scar tissue on the conjunctival surface of the eyelid in old cases with bluish congested patches, and white lines.

I should like to relate a case which shows how long a case of trachoma may last, and that the lymphoid bodies may be concealed under the thickened conjunctiva. A Polish Jew, aged about 30 years, had suffered from his eyes from the time of his arrival in this country when aged about 12 years. He had had frequent attacks of inflammation of the eyes relieved by treatment

but never entirely cured. When I first saw him: on everting his upper eyelids I found the conjunctiva bluish and congested in patches with white lines of cicatrices, and no granulations. In the fornices were rolls of wavy semi-translucent looking conjunctiva; in the right eye there was a small facetted ulcer of the cornea with a clear surface, and some amount of pannus. I applied the acetic acid treatment to the wavy-looking conjunctiva, and followed it with applications of solution of nitrate of silver, 2 per cent., once every week. After several applications he appeared to be cured and I lost sight of him for a few months. He appeared again one day with acute inflammation of the right eye: on everting the eyelid I found a linear swelling extending along almost the whole length of the eyelid. Expecting to find pus I incised the swelling, and uncovered four lymphoid bodies about the size of hemp seeds, which I removed.

I saw this man several times afterwards at long intervals, and found that his eyes gave him no more trouble except a little irritation on windy dusty days which might be expected from the dry cicatricial condition of his conjunctiva.

INHIBITING EFFECT OF ACETIC ACID UPON MICROORGANISMS IN THE PRESENCE OF SODIUM CHLORIDE AND SUCROSE[1,2]

A. S. LEVINE AND C. R. FELLERS

Massachusetts Agricultural Experiment Station, Amherst, Mass.

Received for publication January 27, 1940

Vinegar has long been added to foods in the home and in the commercial packing plant as a flavoring element; in addition, it has a definite preservative action against spoilage microorganisms. Any toxic effect of vinegar is undoubtedly due to its acetic acid content.

Levine and Fellers (1940) showed that the toxicity of acetic acid is not due to pH alone although an increase in the hydrogen ions resulted in a decrease in heat resistance of the bacteria studied.

As vinegar is usually associated with sugar or salt or both in a food, it seemed advisable to study the toxic effect of acetic acid in the presence of these substances.

LITERATURE REVIEW

The uses of vinegar in foods are legion: mayonnaise dressings, prepared mustard, horse-radish, pickles, salads, marinated fish, spinach, and beets are only a few of the products with which it is used. Rowse (1923) attributed the keeping qualities of mayonnaise to its vinegar content and pointed out that the preserving power of vinegar varies directly with its acetic acid content. Pederson and Breed (1926) found that one per cent acetic acid

[1] Contribution 261, Massachusetts Agricultural Experiment Station, Amherst. Taken from Doctor's Dissertation of A. S. Levine (1939), Massachusetts State College.

[2] Presented at the Forty-third General Meeting of the Society of American Bacteriologists, New Haven, Conn. December 29, 1939. J. Bact. 39, 17.

stopped the growth in tomato catsup of all microorganisms studied and that combinations of salt and sugar with the acid did not lower appreciably the amount of acetic acid required.

The antiseptic action of acetic acid and some other organic acids is not confined to bacteria; these acids appear to be toxic to yeasts and molds as well. Cruess and Hascal (1924) reported that 0.8 to 1.0 per cent acetic acid seemed to inhibit the fermentation of apple cider by Burgundy yeast. Katagiri (1928) found that at a constant concentration of free acid, the rate of fermentation by yeast was independent of the concentration of either acetate or formate salts. Similarly, the nature of the salt cation, Na, K, or NH_4 had no influence. The yeast fermentation was, however, very sensitive to the presence of free acid. This investigator states that the effect is no doubt a complex one, due in part to hydrogen ions and in part to the undissociated molecules of the acid but that the influence of the latter is much greater than that of the former.

Fabian and Wadsworth (1939) demonstrated that acetic acid is a better preserving agent for sweet pickles than lactic acid. The pH value of the pickle syrup was not a reliable indicator of the preserving power of the acids present. Levine and Fellers (1940) conducted comparative studies and showed that acetic acid was more toxic than either lactic or hydrochloric acid to *Salmonella aertrycke*, *Saccharomyces cerevisiae*, and *Aspergillus niger*. These organisms were inhibited or destroyed at a higher pH value when acetic acid was used as the toxic agent than where lactic or hydrochloric acids were used.

EXPERIMENTAL

Index organisms

The test microorganisms used were *Salmonella aertrycke*, *Staphylococcus aureus*, *Phytomonas phaseoli*, *Bacillus cereus*; *Saccharomyces cerevisiae*, (Lister strain) and *Aspergillus niger*. These microorganisms were selected, not only for their association with food spoilage, but also for their representative value as more or less typical members of certain microbial groups.

Methods

The effect of acetic acid on the total destruction and on the reduction of numbers of bacteria and yeast was determined by a 15-minute contact with acetic acid solutions. The test microorganisms were also subjected to contact with acetic acid solutions containing 5 per cent salt and with acidified 20 per cent sugar syrups. The solutions were prepared as follows: Five milliliters of sterile distilled water were added by pipette to each of a series of sterile tubes. To the first tube, were added 5 ml. of acetic acid of known concentration. After mixing, 5 ml. of this solution were transferred to the next tube. The procedure was repeated until each tube in the series contained 5 ml. of acid solution in decreasing order of concentration, 5 ml. from the last tube having been discarded. One-tenth ml. of a diluted broth culture was added to each tube. The tubes were well agitated and replaced in the rack for 15 minutes. After this contact period a standard quantitative plating was made on each tube. Nutrient agar was used for the bacteria and glucose agar for the yeast.

The same general procedure was followed where acid-sugar syrups and acid-salt brines were used. Five milliliters of sterile double strength salt brine or sugar syrup were added to the first tube so that dilution with an equal volume of acetic acid gave the same strength brine or syrup in this tube as in the others of the series.

Immediately after plating, determination of pH was made on each solution remaining in the tubes. All pH determinations were made electrometrically by means of a Beckman glass electrode pH meter in which the electrode was balanced against a standard cell. Total acidities were calculated, since the acid concentration of the first tube and the subsequent dilutions were known. These calculations were checked occasionally by actual titration with standardized sodium hydroxide.

The mold was cultivated in flasks of 0.5 per cent glucose broth alone and also in glucose broth with 5 per cent salt and with 20 per cent sugar. The broth solutions in each series were acidified with acetic acid. An unacidified control was used in each series also.

The technique employed in the study of *Aspergillus niger* was somewhat different from that used with bacteria and yeast due to the nature of the mold. Sterile 50 ml. portions of 0.5 per cent glucose broth were transferred aseptically to sterile 250 ml. Erlenmeyer flasks. This procedure was used to obviate unequal loss of flask contents through evaporation during sterilization. The sterile sugar- and salt-glucose broths were added to series of flasks in the same way. Varying amounts of acetic acid were added to the flasks to make series of decreasing acid concentrations. The volume of acid added was sufficiently small to affect the surface of the medium but little. Ten milliliters of solution were removed from each flask for pH and total acidity determinations. Acidity was determined by titration with sodium hydroxide with phenolphthalein as the indicator. The remaining 40 ml. gave a maximum surface in each flask for mold growth. A 0.1 ml. water suspension of spores of *Aspergillus niger* was inoculated into each flask. The flasks were held for five days at 30°C. (86°F.) in the absence of light. The dried weight of mold which had developed during the incubation period was determined by filtering and washing the flask contents. The filtrate was saved for pH and total acidity determinations. The mold mats were dried at 100°C. (212°F.) to a constant weight.

Experimental results

Results are presented in tables 1 to 6. The percentage reduction in whole numbers is based on the number of organisms surviving after a 15-minute contact period with similar solution, water, brine, or syrup, that contained no added acetic acid. Thus, in table 1 at a pH 3.3 and a total acidity of 0.15 per cent acetic acid, there was a 98 per cent reduction in the number of cells of *Salmonella aertrycke* surviving from the number obtained when water at pH 6.2 was used as the contact medium. For brine of the same total acidity, 0.15 per cent, the pH was 3.1 and the reduction was 100 per cent. A 20 per cent sucrose syrup that contained 0.15 per cent acetic acid and had a pH of 3.5 caused a reduction from the number of organisms surviving in the unacidified syrup of 99 per cent. Greater numbers of *Salmonella ae-*

trycke survived contact with the unacidified 5 per cent brine and the unacidified 20 per cent syrup than with the water alone. Bushnell (1921) found that broth media containing 4 per cent NaCl

TABLE 1

Effect of different concentrations of acetic acid on the survival of Salmonella aertrycke in water, salt brine, and sugar solutions

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT	REDUCTION IN NUMBERS
	pH	Calculated total acidity		
		per cent	*bacteria per ml.*	*per cent*
Water + acetic acid	3.3	0.15	1,870	98
Water + acetic acid	3.4	0.08	4,800	94
Water + acetic acid	3.6	0.04	8,400	90
Water + acetic acid	3.8	0.02	13,800	84
Water + acetic acid	4.0	0.01	24,000	71
Water + acetic acid	4.2	0.005	36,000	57
Water only	6.2	0.0	84,000	
5 per cent brine + acetic acid	3.1	0.15	0	100
5 per cent brine + acetic acid	3.3	0.08	2	99+
5 per cent brine + acetic acid	3.5	0.04	6,000	94
5 per cent brine + acetic acid	3.7	0.02	14,400	85
5 per cent brine + acetic acid	4.0	0.01	48,000	50
5 per cent brine + acetic acid	4.3	0.005	84,000	14
5 per cent brine only	6.1	0.0	96,000	
20 per cent syrup + acetic acid	3.5	0.15	1,800	99
20 per cent syrup + acetic acid	3.7	0.08	7,200	94
20 per cent syrup + acetic acid	4.0	0.04	15,600	87
20 per cent syrup + acetic acid	4.4	0.02	21,600	82
20 per cent syrup + acetic acid	4.7	0.01	72,000	40
20 per cent syrup + acetic acid	5.3	0.005	108,000	10
20 per cent syrup only	7.1	0.0	120,000	

102,000 bacteria were added to each ml. of the solution.

were more favorable for growth of the organisms studied than broth containing one per cent. At the higher total acid concentrations, the brine solution was slightly more effective than the water or the syrup solutions in reducing the number of cells of *Salmonella aertrycke*. However, at the lower acid concentrations

the brine seemed to be less effective than the water of corresponding total acidity, in reducing the number of organisms. The

TABLE 2

Effect of different concentrations of acetic acid on the survival of Staphylococcus aureus in water, salt brine, and sugar solutions

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT	REDUCTION IN NUMBERS
	pH	Calculated total acidity		
		per cent	*bacteria per ml.*	*per cent*
Water + acetic acid	3.3	0.17	17	99+
Water + acetic acid	3.4	0.08	114	99
Water + acetic acid	3.6	0.04	260	97
Water + acetic acid	3.8	0.02	2,400	76
Water + acetic acid	4.0	0.01	5,000	50
Water + acetic acid	4.2	0.005	9,200	8
Water + acetic acid	4.4	0.003	10,800	
Water only	6.2	0.0	10,000	
5 per cent brine + acetic acid	3.1	0.17	0	100
5 per cent brine + acetic acid	3.2	0.08	14	99+
5 per cent brine + acetic acid	3.4	0.04	315	93
5 per cent brine + acetic acid	3.6	0.02	1,200	83
5 per cent brine + acetic acid	3.7	0.01	1,500	79
5 per cent brine + acetic acid	4.0	0.005	4,200	42
5 per cent brine + acetic acid	4.2	0.003	6,400	11
5 per cent brine only	6.1	0.0	7,200	
20 per cent syrup + acetic acid	3.5	0.17	0	100
20 per cent syrup + acetic acid	3.7	0.08	1	99+
20 per cent syrup + acetic acid	4.0	0.04	3	99+
20 per cent syrup + acetic acid	4.3	0.02	61	99
20 per cent syrup + acetic acid	4.7	0.01	900	79
20 per cent syrup + acetic acid	5.1	0.005	3,600	14
20 per cent syrup + acetic acid	5.9	0.003	6,000	
20 per cent syrup only	7.1	0.0	4,200	

7,200 bacteria were added to each ml. of the solution.

brine was also less effective than the syrup at equivalent pH but this is not surprising inasmuch as the syrup contained a slightly higher percentage of the acetic acid.

Table 2 shows that plain water reduced the numbers of *Staphy-*

Micrococcus aureus less than did the unacidified salt or sugar solutions. At the high concentrations of acid, all three solutions were about equally effective in reducing the numbers of added viable bacteria. At pH 4.2 the percentage reduction was approximately the same in the water and in the 5 per cent salt brine solutions, being 8 and 11 per cent, respectively. However, at pH 4.3 in the 20 per cent sugar syrup series, the number of living cells was reduced 99 per cent. In this case some of the reduction may be attributed to the slightly higher acid concentration at this pH value. The syrup permitted the survival of no organisms at pH 3.5 and acidity of 0.17 per cent, whereas at pH 3.3 and the same total acidity, the water solution permitted the survival of some organisms.

Table 3 presents the results obtained on an experiment of a similar nature with *Phytomonas phaseoli*, a plant pathogen. This organism proved to be less resistant to acetic acid than did the other microorganisms. Water and acetic acid at pH 3.4, and brine and syrup at pH 4.0 caused a 100 per cent destruction of the organisms in 15 minutes. In addition, both the brine and the syrup controls with no added acid were more toxic than the water alone. Again, the acidified sugar syrup at any pH caused a greater reduction in numbers than did the brine or water series solution of a corresponding hydrogen-ion concentration. This was true, despite the fact that the unacidified sugar syrup was less toxic to *Phytomonas phaseoli* than the plain salt brine.

Bacillus cereus, an organism closely related to *Bacillus mesentericus* which causes a "ropy" spoilage in many foods, yielded the results in table 4. The unacidified 20 per cent sugar solution seemed to enhance the survival of *Bacillus cereus*, whether as vegetative cells or as spores. But, the addition of acid to the syrup again caused a greater reduction in numbers of surviving organisms than that obtained after contact with brine or water solutions of a corresponding pH value. Although the brine alone was slightly more toxic than the water alone, the latter, when acidified with acetic acid, caused a greater reduction in numbers than the former at the same total acid concentration. If one compares the results on corresponding hydrogen-ion concentration, the difference becomes more marked due, probably, to the

higher acid concentration in the water solution. The same trend, in general, is observed in the case of spores although there are a

TABLE 3

Effect of different concentrations of acetic acid on the survival of Phytomonas phaseoli in water, salt brine, and sugar solutions

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT	REDUCTION IN NUMBERS
	pH	Calculated total acidity		
		per cent	*bacteria per ml.*	*per cent*
Water + acetic acid	3.3	0.15	0	100
Water + acetic acid	3.4	0.08	0	100
Water + acetic acid	3.6	0.04	41	99+
Water + acetic acid	3.8	0.02	69	99
Water + acetic acid	4.0	0.01	167	98
Water + acetic acid	4.2	0.005	5,000	50
Water + acetic acid	4.4	0.002	6,000	40
Water only	6.2	0.0	10,000	
5 per cent brine + acetic acid	3.1	0.15	0	100
5 per cent brine + acetic acid	3.3	0.08	0	100
5 per cent brine + acetic acid	3.5	0.04	0	100
5 per cent brine + acetic acid	3.7	0.02	0	100
5 per cent brine + acetic acid	4.0	0.01	0	100
5 per cent brine + acetic acid	4.3	0.005	30	98
5 per cent brine + acetic acid	4.6	0.002	440	68
5 per cent brine only	6.1	0.0	1,380	
20 per cent syrup + acetic acid	3.5	0.15	0	100
20 per cent syrup + acetic acid	3.7	0.08	0	100
20 per cent syrup + acetic acid	4.0	0.04	0	100
20 per cent syrup + acetic acid	4.4	0.02	24	99+
20 per cent syrup + acetic acid	4.7	0.01	900	86
20 per cent syrup + acetic acid	5.3	0.005	4,200	36
20 per cent syrup + acetic acid	6.5	0.002	5,400	18
20 per cent syrup only	7.1	0.0	6,600	

7,200 bacteria were added to each ml. of the solution.

smaller number of organisms involved. Since acetic acid appears to be as effective against spores as against vegetative cells, it is significant that it may be useful for the control of spore-forming bacteria in foods.

Yeasts can generally tolerate higher acidities than most bacteria. Table 5 shows that *Saccharomyces cerevisiae* survived contact with acidified water, brine, and syrup solutions at higher total

TABLE 4

Effect of different concentrations of acetic acid on the survival of Bacillus cereus in water, salt brine, and sugar solutions

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT; BACTERIA + SPORES	REDUCTION IN NUMBERS	TOTAL COUNT; SPORES ONLY	REDUCTION IN NUMBERS
	pH	Calculated total acidity				
		per cent	*per ml.*	*per cent*	*per ml.*	*per cent*
Water + acetic acid	3.3	0.17	68	79	5	93
Water + acetic acid	3.4	0.08	85	74	17	76
Water + acetic acid	3.6	0.04	94	71	16	77
Water + acetic acid	3.8	0.02	99	70	17	76
Water + acetic acid	4.0	0.01	125	62	21	70
Water + acetic acid	4.2	0.005	150	55	27	61
Water only	6.2	0.0	330		70	
5 per cent brine + acetic acid	3.1	0.17	80	68	4	93
5 per cent brine + acetic acid	3.2	0.08	95	62	13	78
5 per cent brine + acetic acid	3.4	0.04	98	63	15	75
5 per cent brine + acetic acid	3.6	0.02	101	60	16	73
5 per cent brine + acetic acid	3.7	0.01	140	44	25	58
5 per cent brine + acetic acid	4.0	0.005	190	24	26	57
5 per cent brine only	6.1	0.0	250		60	
20 per cent syrup + acetic acid	3.5	0.17	95	87	10	87
20 per cent syrup + acetic acid	3.7	0.08	108	85	17	78
20 per cent syrup + acetic acid	4.0	0.04	114	84	20	75
20 per cent syrup + acetic acid	4.3	0.02	126	83	20	75
20 per cent syrup + acetic acid	4.7	0.01	150	79	22	72
20 per cent syrup + acetic acid	5.1	0.005	210	71	25	68
20 per cent syrup only	7.1	0.0	720		70	

800 bacteria and spores were added to each ml. of the solution.

84 spores were added to each ml. of the solution.

acidities and greater hydrogen-ion concentrations than any of the bacteria studied. The acidity range of the solutions was from 1.0 to 4.72 per cent. A comparison of the water series with the brine series again shows that toxicity is due in part to the acetic

acid itself, without the influence of hydrogen-ion concentration. Although the plain brine was slightly more toxic than the water, the acidified brine caused a smaller reduction in surviving cells

TABLE 5

Effect of different concentrations of acetic acid on the survival of Saccharomyces cerevisiae (Lisier strain) in water, salt brine, and sugar solutions

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT	REDUCTION IN NUMBERS
	pH	Calculated total acidity		
		per cent	*yeast cells per ml.*	*per cent*
Water + acetic acid	2.5	4.73	0	100
Water + acetic acid	2.7	2.37	78	88
Water + acetic acid	2.8	1.18	300	55
Water + acetic acid	3.0	0.59	420	37
Water + acetic acid	3.1	0.30	470	30
Water + acetic acid	3.3	0.15	500	25
Water only	6.2	0.0	670	
5 per cent brine + acetic acid	2.2	4.73	0	100
5 per cent brine + acetic acid	2.4	2.37	20	97
5 per cent brine + acetic acid	2.6	1.18	370	38
5 per cent brine + acetic acid	2.7	0.59	440	27
5 per cent brine + acetic acid	2.9	0.30	410	32
5 per cent brine + acetic acid	3.0	0.15	450	25
5 per cent brine only	6.1	0.0	600	
20 per cent syrup + acetic acid	2.5	4.73	0	100
20 per cent syrup + acetic acid	2.7	2.37	15	97
20 per cent syrup + acetic acid	2.9	1.18	280	44
20 per cent syrup + acetic acid	3.1	0.59	390	22
20 per cent syrup + acetic acid	3.3	0.30	470	6
20 per cent syrup + acetic acid	3.6	0.15	490	2
20 per cent syrup only	7.1	0.0	500	

900 yeast cells were added to each ml. of the solution.

than did the acidified water at a corresponding pH. Thus, at pH 3.0 the brine caused a reduction of 25 per cent whereas a 15-minute contact period with water at this pH resulted in a reduction of 37 per cent. Yet the water solution at pH 3.0 had a

total acidity of 0.59 per cent while the brine at the same pH had only 0.15 per cent acidity. The greater toxicity of the water solution seems to be due, therefore, to the higher content of acetic

TABLE 6

Effect of different concentrations of acetic acid on the growth of Aspergillus niger in flasks containing glucose broth, glucose broth brine, and glucose broth syrup

MEDIUM	INITIAL ACIDITY		FINAL ACIDITY		DRIED MOLD WEIGHT
	pH	Titratable acidity	pH	Titratable acidity	
		per cent		*per cent*	*mgm.*
Glucose broth	4.1	0.27	4.1	0.27	0
Glucose broth	4.3	0.21	4.3	0.21	17
Glucose broth	4.4	0.16	7.1	0.03*	162
Glucose broth	4.5	0.15	7.6	0.03	148
Glucose broth	4.7	0.10	7.6	0.03	133
Glucose broth	5.2	0.07	7.6	0.03	121
Glucose broth (control)	6.8	0.03	7.6	0.03	123
Glucose broth + 5 per cent NaCl	4.0	0.27	3.9	0.32	0
Glucose broth + 5 per cent NaCl	4.1	0.22	4.2	0.18	33
Glucose broth + 5 per cent NaCl	4.2	0.17	7.5	0.03*	132
Glucose broth + 5 per cent NaCl	4.5	0.12	7.5	0.03	127
Glucose broth + 5 per cent NaCl	4.6	0.10	7.6	0.03	129
Glucose broth + 5 per cent NaCl (control)	6.7	0.03	7.6	0.03	131
Glucose broth + 20 per cent sucrose	4.1	0.27	4.1	0.27	0
Glucose broth + 20 per cent sucrose	4.2	0.22	4.2	0.20	6
Glucose broth + 20 per cent sucrose	4.3	0.20	3.2	0.49	64
Glucose broth + 20 per cent sucrose	4.4	0.16	2.2	2.47	403
Glucose broth + 20 per cent sucrose	4.7	0.11	2.3	2.33	327
Glucose broth + 20 per cent sucrose (control)	6.8	0.03	2.1	3.02	223

The inoculation consisted of 0.1 ml. of a water suspension of *Aspergillus niger* which contained 2000 spores.

* At final pH values of more than 7.0 the apparent acidity represents a blank which is not subtracted in this table.

acid as it could not be due to any difference in the concentration of hydrogen-ions. Bach (1932) stated that generally the hydrogen-ions control antiseptic effect but that the undissociated part of lactic acid is the active factor when pH is such as to be unimportant.

The data in table 6 show the effect of different concentrations of acetic acid on the growth of *Aspergillus niger* in glucose broth, glucose broth with 5 per cent sodium chloride salt, and glucose broth with 20 per cent sucrose. The brine control and the glucose broth control yielded about the same amount of dried mold, 131 and 128 mgm., respectively. However, growth in glucose broth control containing 20 per cent sucrose yielded a dried mold mat weighing 223 grams. This mold growth was sufficient to form 3.02 per cent acid from the sucrose and increase the hydrogen-ion concentration to pH 2.1. The acid produced is calculated as acetic acid for purposes of comparison, although *Aspergillus niger* readily produces citric, oxalic, and gluconic acids when grown in sugar solution. Henrici (1930) states that on prolonged incubation the molds slowly utilize the acids they form thus causing the reaction to return toward neutrality. This is probably what occurred in the acidified broth where sugar was not present and which had mold growth. The acidities were completely utilized from the start and the reaction went beyond the neutral point. In all three series, 0.27 per cent acetic acid, or more, was necessary to prevent the growth of mold. Also, inhibition was about the same in all flasks containing 0.21 and 0.22 per cent acid as 17, 6, and 33 mgm. may be considered as equal insofar as dried mold weight is concerned (see fig. 1). Kirby, Frey, and Atkin (1935) found that the growth of *Aspergillus niger* was inhibited in a bread medium set at pH 3.5 and which contained 0.2 per cent acetic acid.

The heaviest mold mat obtained (see table 6), developed in the 0.5 per cent glucose broth containing 20 per cent sucrose and 0.16 per cent acid with a pH of 4.4. The dried mold weighed 463 mgm. A titratable acidity of 2.47 per cent with pH 2.2 was produced in the medium. In general, the sugar-broth series promoted greater mold development than did either of the other two series at corresponding acidities. The mold growths in the glucose brine series and in the plain glucose series were about the same. At a total acidity of 0.17 per cent, the former yielded 182 mgm. of dried mold whereas the latter at a total acidity of

0.16 per cent yielded 162 mgm. Yet the salted broth had a pH 4.2 which was lower than the pH 4.4 of the 0.16 per cent plain broth solution. This might have inhibited initial growth of the mold sufficiently to account for the difference, as a pH value of 4.2 is close to the inhibiting limit of acetic acid for *Aspergillus niger*. At pH 4.5 the plain glucose broth had an acidity of 0.15

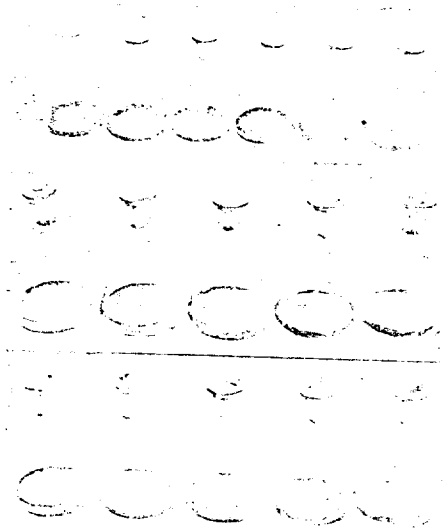


FIG. 1. Growth of *Aspergillus niger* in flasks of glucose broth with added acetic acid (top row); with acetic acid and 5 per cent NaCl (middle row); and with acetic acid and 20 per cent sucrose (bottom row).
Top row: percentage acetic acid, left to right: none, 0.07, 0.10, 0.16, 0.21, 0.27.
Middle row: percentage acetic acid, left to right: none, 0.10, 0.17, 0.23, 0.27.
Bottom row: percentage acetic acid, left to right: none, 0.11, 0.16, 0.22, 0.27.

per cent and yielded 148 mgm. of dried mold. At a pH 4.5 the glucose broth brine had an acidity of 0.12 per cent and yielded 127 mgm. of dried mold. This small difference in mold weight might be attributed to the higher content of acid available for the mold in the plain broth medium, as the hydrogen-ion concentration in this case was not sufficient to cause any inhibition of growth during the first part of the incubation period.

SUMMARY

1. Acetic acid inhibited bacterial growth in almost direct proportion to the amount present. The order of decreasing resistance to acetic acid, either alone, in 5 per cent brine, or in 20 per cent sugar syrup is *Bacillus cereus*, *Salmonella aertrycke*, *Staphylococcus aureus*, and *Phytomonas phaseoli*. Naturally, *Saccharomyces cerevisiae* and *Aspergillus niger* were inhibited at a higher acetic acid concentration than the bacteria, with the yeast being the more resistant.

2. At equivalent pH values greater toxicity was usually observed in those tubes containing the greater amount of acid. Apart from the indirect effect in altering the hydrogen-ion concentration, the salt and the sugar aided the acetic acid but little in its toxic effect on bacteria and yeast. Similarly, the added salt and sugar exerted little, if any, effect on the minimum percentage acidity required for total destruction of these organisms.

3. Additional evidence is presented to show that the toxic action of acetic acid on microorganisms is not confined to the hydrogen-ion concentration alone (Levine and Fellers, 1940).

4. When *Aspergillus niger* was inoculated into a series of flasks of glucose broth containing different acetic acid concentrations, growth was inhibited at pH 4.1 and at a total acidity of 0.27 per cent. The addition of either 5 per cent salt or 20 per cent sucrose did not significantly change these limits for growth. The presence of acetic acid in non-toxic amounts actually promoted the development of *Aspergillus niger* by acting as a source of energy.

5. At acetic acid concentrations of 0.10 to 0.17 per cent no inhibition in mold growth resulted from the addition of 5 per cent NaCl. On the other hand the addition of 20 per cent sucrose markedly stimulated growth. Maximum mold growth was obtained in a 20 per cent nutrient sucrose solution at pH 4.4 containing 0.16 per cent acetic acid.

REFERENCES

Bach, D. 1932 The antiseptic action of organic acids on some bacteria. Bull. sci. pharmacol., 39, 7–23. Chem. Abstracts, 26, 2210.

Bushnell, L. D. 1921 Influence of vacuum upon growth of some aerobic spore-bearing bacteria. J. Bact., 7, 283–300.

CRUESS, W. V., AND HASCAL, R. 1924 Comparative effects of acetic acid and vinegar bacteria on fermentation. Fruit Products J., 3, no. 6, 20-21.

FABIAN, F. W., AND WADSWORTH, C. K. 1930 Experimental work on lactic acid in preserving pickles and pickle products. II. Preserving value of acetic and lactic acids in the presence of sucrose. Food Research, 4, 511-519.

HENRICI, A. T. 1930 Molds, Yeasts, and Actinomycetes. 296 pp., John Wiley and Sons, Inc., New York.

KATAGIRI, H. 1926 The influence of fatty acids and their salts on alcoholic fermentation by living yeast. I. Acetic and formic acids and their sodium, potassium, and ammonium salts. Biochem. J., 20, 427-436.

KIDDY, G. W., FREY, C. N., AND ATKIN, L. 1935 The growth of bread molds as influenced by acidity. Cereal Chem., 12, 244-255.

LEVINE, A. S., AND FELLERS, C. R. 1940 Action of acetic acid on food spoilage microorganisms. J. Bact., 39, 499-515.

PEDERSON, C. S., AND BREED, R. S. 1926 Preservative action in catsup of salt, sugar, benzoate, and acid. N. Y. Agr. Expt. Sta. Bull., 533, 15 pp.

ROWSE, A. E. 1923 A talk on vinegar. Fruit Products J., 8, no. 3, 15.

J. Dairy Sci. 50(7): 1153-1157, 1967

Absorption and Utilization of Volatile Fatty Acids by the Young Calf[1]

Y. T. LIANG, J. L. MORRILL, and J. L. NOORDSY
Departments of Dairy and Poultry Science and Surgery and Medicine
Kansas State University, Manhattan

Abstract

Absorption and utilization of volatile fatty acids (VFA) by young milk-fed calves were studied using sodium salts of ^{14}C-labeled acids. In two trials acetate was administered orally; in four later trials, either acetate, propionate, or n-butyrate was administered into the extreme terminal portion of the ileum via a re-entrant fistula, followed several hours later by the same acid administered orally. Radioactivity was measured in expired CO_2, ileal digesta, feces and, in one experiment, blood.

Radioactivity in blood of a week-old calf peaked 90 min after acetate feeding. In two other acetate trials radioactivity in expired CO_2 peaked 3 hr after feeding. Peak activity in expired CO_2 appeared in 2 hr or less after ileal administration. Large amounts of the ^{14}C were eliminated as $^{14}CO_2$, primarily during the first 8 hr.

Separate trials with propionate and butyrate indicated they also were efficiently absorbed and used.

In all trials, only small amounts of ^{14}C appeared in feces, urine, or ileal digesta. These data indicate that the very young calf can absorb and metabolize VFA, whether the acids are produced in the forestomach or large intestine.

Volatile fatty acids (VFA) are important nutrients for mature ruminants. Recently, Leng and Leonard (1) estimated that VFA utilization could account for about 79% of the energy required by sheep. In contrast to the large amount of research concerning VFA produced in the forestomach of the bovine, little attention has been directed toward production and utilization of VFA originating in the large intestines. Various workers (3, 10, 11) have reported concentrations of VFA in intestines of animals of various age fed different diets; however, information is scarce on the ability of the young calf to absorb VFA from the posterior gastrointestinal tract. This research was conducted to determine the extent to which VFA are absorbed from the large intestine of young milk-fed calves and how efficiently these acids are used.

Received for publication February 27, 1966.

[1]Contribution No. [illegible] Department of Dairy and Poultry Science, [illegible] Department of Surgery and Medicine, Kansas Agricultural Experiment Station, Manhattan.

Experimental Procedure

Utilization of the sodium salts of acetic, propionic, and n-butyric acids was studied using six male dairy calves. The calves were separated from their dams, when three days old, and transferred to a metabolism crate. Except during experimental periods, they were fed only whole milk supplemented with vitamins A, D, and E, trace minerals, and oxytetracycline. Breeds and ages of the calves are shown in Table 1. Four calves (Trials 3-6) were fitted with re-entrant ileal fistulae at the posterior end of the ileum by the method of Noordsy and Morrill (8) one week before the experiment started. The salts, labeled at carbon 1, were administered either orally or by oral-intestinal combination (Table 1).

For oral administration, the isotope was mixed with milk fed from a nipple pail. For intestinal administration, the cannulae were disconnected and the salt solution was poured into the distal cannula. To keep the acid out of the small intestine, the cannulae were not reconnected for an hour. During the hour ileal digesta were manually collected and poured into the distal cannula.

Collection of samples. Acid utilization was estimated by measuring radioactivity in blood, feces, urine, expired CO_2, intestinal contents, or a combination of these (Table 1). Blood samples were collected from an external jugular vein. Feces and intestinal digesta, collected by the methods described by Das (2), were frozen until analyzed. Urine excreted was measured and aliquots were saved for analysis.

Expired CO_2 was measured as follows: Immediately prior to the start of the experiment, the calves were fitted with an endotracheal tube through a tracheal incision. The exposed end of the tube was connected to a Precision wet test gas meter through a valve that allowed fresh air intake and directed expired air

TABLE 1

Experimental animals, design and per cent of administered ^{14}C recovered

Trial	Animal Age	Animal Breed	Sodium salt administered	Route and amount of administered ^{14}C		Per cent administered ^{14}C recovered in samples	
	(days)				*(μc)*		
1	8	Holstein	Acetate-1-^{14}C	Oral	1,022	Blood	Not determined
						Feces	3.8 (240)[a]
						Urine	1.5 (240)
2	8	Holstein	Acetate-1-^{14}C	Oral	1,024	Exhaled CO_2	57 (29)
						Feces	1.6 (45)
						Urine	1.0 (45)
3	14	Holstein	Acetate-1-^{14}C	Oral	600	Exhaled CO_2	52 (22)
				Ileal	500	Ileal digesta	3.8 (26)[b]
						Feces	2.5 (35)
						Urine	0.75 (35)
4	19	Ayrshire	n-Butyrate-1-^{14}C	Oral	514	Exhaled CO_2[c]	
				Ileal	412	Ileal digesta	1.3 (45)[b]
						Feces	2.7 (264)
						Urine	1.5 (47)
5	21	Ayrshire	Propionate-1-^{14}C	Oral	540	Exhaled CO_2[c]	
				Ileal	432	Ileal digesta	6.24 (16)[b]
						Feces	0.6 (216)
						Urine	1.5 (20)
6	20	Holstein	Acetate-1-^{14}C	Ileal	464 (2 %)	Exhaled CO_2	Not determined

[a] Values in parentheses indicate the number of hours during which measurements were made.
[b] Per cent of the orally administered ^{14}C that passed through the fistula.
[c] Explained in text.

through the gas meter. A collection device was attached to the gas meter.

Analyses. Radioactivity in samples was determined by counting in a Packard Tri-Carb Model 3214 Liquid Scintillation Spectrometer. Background counts and efficiency of counting in various samples were determined using internal standards.

Carbon dioxide from the collected samples of expired air was absorbed in 1:1 (v/v) monoethanolamine-ethylene glycol monomethyl ether. Two milliliters of that mixture were added to 10 ml toluene scintillator solvent and 7 ml ethylene glycol monomethyl ether to determine radioactivity. The toluene scintillator solvent was 100 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 10 g 2,5-diphenyloxazole (PPO), and enough toluene to make one liter.

Radioactivity in blood samples was determined in dioxane scintillator solvent, following the removal of proteins by the method of Nelson (6). The dioxane scintillator solvent was 60 g naphthalene, 4 g PPO, 200 mg POPOP, 100 ml methanol, 20 ml ethylene glycol, and enough dioxane to make one liter. Urine samples were diluted with water and added to dioxane solvent for counting.

Feces and ileal digesta samples were oven dried at 130 C in an air tight container connected, by glass tubing, to a collection trap outside the oven. The evaporated fraction was assayed in dioxane solvent. The dried sample was combusted in a jar filled with oxygen; the carbon dioxide was absorbed and assayed as expired air samples were.

Results

Absorption and utilization of acetate. Trial 1 was conducted to determine whether acetate fed to the young milk-fed calf was absorbed or excreted in feces. The data (Table 1, Figure 1) indicate that a high percentage was rapidly absorbed. Radioactivity in the blood peaked 1.5 hr after feeding, and only 3.8% of the administered acetate appeared in the feces during the next ten days. The low activity in urine during those ten days is additional indirect evidence for efficient use of the absorbed acetate.

Trial 2 was to confirm the high acetate utilization indicated in Trial 1. Radioactivity in expired CO_2 peaked 3 hr after feeding (Figure 1). As in Trial 1, urine radioactivity was low. Because of respiratory difficulty, the endotracheal tube was removed from the calf after

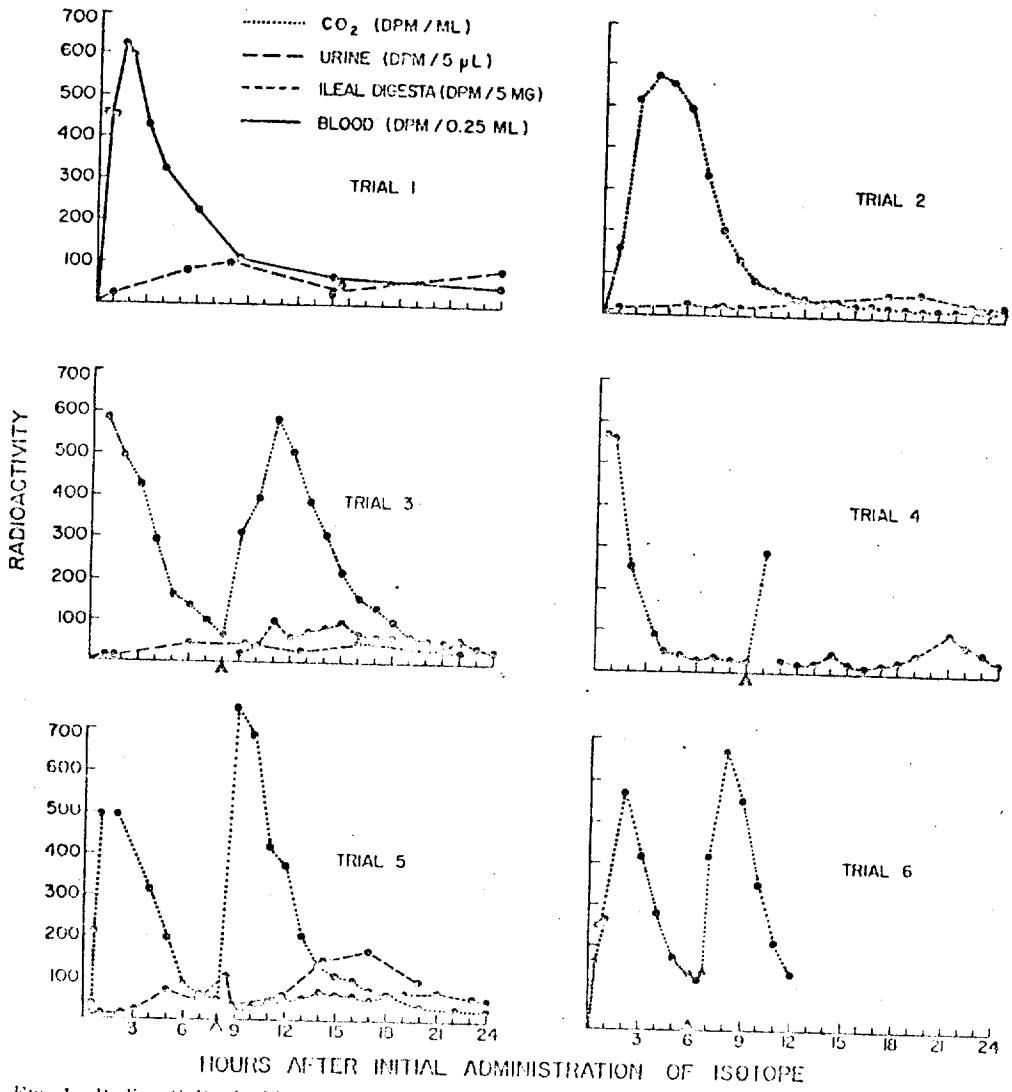


FIG. 1. Radioactivity in biological samples at indicated times after salts of ^{14}C-labeled volatile fatty acids were administered. ▲ Indicates second administration of isotope.

29 hr of the experiment. During the 29 hr, 57% of the administered labeled carbon was recovered in the exhaled air, mostly during the first 12 hr (Figure 1). Only 2.6% of the radioactivity was excreted in the feces and urine during the 45 hr experiment (Table 1).

Results of Trials 1 and 2 indicate that orally fed acetate can be absorbed and metabolized by the young calf. To determine the extent of absorption of VFA from the large intestine, after Trial 2 radioactive salts were administered into the distal end of the re-entrant ileal fistula. Results of Trial 3 (Table 1, Figure 1), in which acetate was orally fed 8 hr after ileal administration, show that the acetate was rapidly absorbed both anterior and posterior to the ileum terminal. The initial sample of exhaled air, taken 1 hr after ileal administration, contained more activity than any other sample, so peak activity may have been earlier. In this trial, collecting and sampling of ileal digesta were begun after the isotope was orally administered. Only 3.8% of the orally fed ^{14}C passed through the fistula the first 29 hr after feeding, compared with an estimated 62% exhaled during the first 11 hr. (This estimation

was made by extrapolating the curve for ileal administration and subtracting these values from the curve for oral administration.)

Absorption and utilization of butyrate and propionate. Trial 4, except for using sodium n-butyrate-1-^{13}C, was similar to Trial 3. The calf had difficulty in breathing early in the trial, so measurement of volume of exhaled air was discontinued. Expired air was sampled by connecting the endotracheal tube directly to the sampler, and radioactivity was determined as in previous trials. Two hours after the isotope was fed, the endotracheal tube became occluded and was removed. No attempt was made to calculate the percentage of administered isotope in expired air. Volumes of air exhaled by calves of similar age and size and maintained under similar environmental conditions should be approximately the same. If so, the curve for expiration of ^{13}C in Trial 4 (Figure 1) is validly comparable to expiration curves from previous trials. The pattern for absorption and utilization of n-butyrate appears to be similar to that of acetate when the VFA is administered into the large intestine. From the one sample of air collected after feeding and the amount recovered in ileal digesta during the 45 hours after feeding (13% of that fed), it appears that orally fed n-butyrate also was absorbed rapidly and to a considerable extent.

In Trial 5, conducted to study utilization of propionate, volume of expired air was not measured during the first day. Samples of exhaled air were collected for analysis as in Trial 4. Radioactivity in expired air increased 15 min after intestinal administration and peaked between 1 and 2 hr (Figure 1). Orally fed propionate was used especially rapidly, with the highest radioactivity in expired air observed 1 hr after feeding. Twenty-four hours after the trial started, the expiration rate of the same calf was measured for 5 hr. Using the average of 213 liters per hour determined during those 5 hr, an estimated 70% of ^{13}C was recovered in expired CO_2 during the first 24 hr. Only a small percentage of the isotope was excreted in urine and feces (Table 1).

In trials thus far discussed, the radioactive carbon was in relatively small amounts of nonradioactive material. Specific activities of the compounds were: (millicuries/millimole) acetate, 2.1; propionate, 2.7; butyrate, 2.9. The possibility existed that the young calf could absorb and metabolize small amounts of VFA but that the amount would be too small to be of any significance. Trial 6 determined the effect that concentration of larger amounts of acetate would have on absorption and metabolism of radioactive VFA in doses of the size previously given. Allowing for the activity remaining from the initial administration, response following administration of 358 mg of nonradioactive sodium acetate with the radioactive acetate was similar to that obtained when the same amount of radioactive acetate was given alone (Figure 1).

Discussion

Since microbial fermentations that occur in the posterior of the intestinal tract are the most likely sources of VFA to young calves fed liquid meals, absorption from that area was of special interest in this study. Results show that the young milk-fed calf is able to rapidly absorb acetic, propionic, and n-butyric acids posterior to the ileocecal junction. These results also indicate rapid metabolism of the VFA, thus confirming results of Young et al. (12). The data presented also show that VFA are absorbed from the stomach or small intestine, or both. Barcroft and co-workers (1) did not find significant amounts of VFA in blood draining the abomasum and small intestines of mature sheep. However, nearly complete absorption of the acids from the rumen, reticulum, and omasum, or the age and species of animal used, might explain their results. Sutton et al. (9) observed inefficient absorption of VFA from the rumen of the milk-fed calf; thus, absorption of VFA is not an inherent property of all areas of the gastrointestinal tract. Their results demonstrated that metabolic activity of the rumen mucosa is important in absorption.

Our data do not indicate whether the butyrate molecule was absorbed directly or underwent previous cleavage, nor do they indicate mechanism of absorption. If the acids were absorbed by passive transport, the butyrate molecule, if it was absorbed unaltered, must approach the maximum size that can be thus absorbed, because glucose is absorbed very slowly, if at all, from the large intestine of the young calf (5).

Not enough information is available to assess the significance of VFA absorbed from the large intestine of young calves. How much energy VFA contributes can be calculated only after fermentation rates have been determined. Besides energy contributed, VFA may also have important physiological functions. For example, data of Young et al. (12), as well as data reported here, show that the young calf has the enzymes necessary to metabolize VFA even before bacterial fermentation begins in the rumen. It is likely, however, that both ruminants and cows absorbed VFA from their large intestines before they were put on experiment.

Also, the effect of VFA, particularly propionate, on the level of blood glucose (12) suggests a relationship between VFA absorbed from the large intestine and the decline in blood glucose that occurs early in a calf's life. Although Nicolai and Stewart (7) showed that the decline occurred without forestomach fermentation, the abomasal feeding they practiced certainly must have resulted in intestinal fermentation.

References

(1) Barcroft, J., McAnally, R. A., and Phillipson, A. T. 1944. Absorption of Volatile Acids from the Alimentary Tract of the Sheep and Other Animals. J. Exptl. Biol., 20: 120.

(2) Das, C. T. 1966. A Study of Digestion of Starch in the Large Intestine of the Young Calf by an in Vitro Technique. M.S. thesis, Kansas State University, Manhattan.

(3) Huber, J. T., and Moore, W. E. C. 1964. Short-Chain Fatty Acid Concentrations Posterior to the Stomach of Calves Fed Normal and Milk Diets. J. Dairy Sci., 47: 1421.

(4) Leng, R. A., and Leonard, G. J. 1965. Measurement of the Rates of Production of Acetic, Propionic, and Butyric Acids in the Rumen of Sheep. Brit. J. Nutrition, 19: 469.

(5) Morrill, J. L., and Liang, Y. T. 1965. Unpublished data.

(6) Nelson, N. 1944. A Photometric Adaptation of the Somogyi Method for the Determination of Glucose. J. Biol. Chem., 153: 375.

(7) Nicolai, J. H., and Stewart, W. E. 1965. Relationship Between Forestomach and Glycemia in Ruminants. J. Dairy Sci., 48: 56.

(8) Noordsy, J. L., and Morrill, J. L. 1965. Unpublished procedure.

(9) Sutton, J. D., McGilliard, A. D., and Jacobson, N. L. 1963. Functional Development of Rumen Mucosa. I. Absorptive Ability. J. Dairy Sci., 46: 426.

(10) Ward, J. K., Richardson, D., and Tsien, W. S. 1961. Volatile Fatty Acid Concentrations and Proportions in the Gastrointestinal Tract of Full-Fed Beef Heifers. J. Animal Sci., 20: 830.

(11) Yang, M. G. 1965. Absorption and Secretion of Nutrients Throughout the Alimentary Tract of Young Calves Fed Different Levels of Fiber and Phosphorus. Dissertation Abstrs., 25: 5175.

(12) Young, J. W., Tove, S. B., and Ramsey, H. A. 1965. Metabolism of Acetate, Propionate, and n-Butyrate in Young Milk-Fed Calves. J. Dairy Sci., 48: 1079.

Preparation and Evaluation of Butter Culture Flavor Concentrates[1,2]

J. Dairy Sci. 50(1): 25-31 (1967)

R. C. LINDSAY, E. A. DAY,[3] and L. A. SATHER
Department of Food Science and Technology, Oregon State University, Corvallis

Abstract

Synthetic butter culture concentrates, based on the analyses of natural high-quality products, were prepared for butter, Cottage cheese, sour cream, and buttermilk. Flavor compounds included in the formulations were diacetyl, acetaldehyde, dimethyl sulfide, acetic acid, and lactic acid. Products flavored with the culture flavor concentrates were found to possess a culture flavor very similar to that produced by natural butter cultures. A student preference panel showed equal preference for high-quality naturally cultured products and artificially flavored products.

The development of sufficient amounts of desirable culture flavor and aroma is one of the major problems facing the cultured dairy products industry today. In recent years a number of methods for providing enhanced culture flavor in Cottage cheese, sour cream, cultured cream butter, and cultured buttermilk have been proposed.

One general approach involves the use of modified cultures and culturing techniques. Some degree of success has been achieved by incorporating high diacetyl-producing strains of *Leuconostoc citrovorum* or *Streptococcus diacetilactis* into general-purpose mixed-strain cultures specifically formulated for use in the manufacture of cultured products. However, experience with such mixed-strain cultures has shown that it is difficult to realize consistency in flavor production under routine plant propagation programs. Alternate natural culturing methods involve the use of pure cultures of *L. citrovorum* (12, 19), or *S. diacetilactis* (18). These methods improve flavor, but the attainment of uniform, desirable culture flavor is not always easily achieved.

Starter distillates have been used with some degree of success for both producing and enhancing culture flavor. However, it is well accepted that they yield a harsh diacetyl flavor and do not duplicate the balanced flavor obtained through use of successful natural culturing procedures. Numerous studies have been conducted on the use of diacetyl as a flavoring agent in cultured dairy products (1, 2, 22). The general conclusion from these studies has been that diacetyl alone imparts a definite culture flavor, but at the same time gives an undesirable harsh flavor.

In previously reported work from this laboratory on the identification of volatile flavor fraction from butter cultures (16), it was noted that most compounds found in butter culture were also present in control heated milk used for culture preparation. The data suggested that the quantitative relationships of the flavor compounds were extremely important in defining culture flavor. This paper relates results of quantitative studies on key culture flavor compounds and the subsequent development of synthetic butter culture flavor formulations.

Experimental Procedure

Preparation of butter cultures. A mixed-strain butter culture of commercial origin, selected for its ability to produce a good cultured cream butter flavor, was employed for culture flavor development. Cultures were propagated in a whole milk medium containing 0.2% added sodium citrate. The milk medium was heated in a boiling-water bath for 1 hr. Mother cultures were transferred every other day, using from 0.6 to 3.0% of inoculum, and the incubation time at 21 C was controlled so that the titratable acidity of the cooled culture was from 0.68-0.78%. Bulk culture was prepared in a similar manner, except that the titratable acidity was allowed to develop to 0.90-0.96%. Bulk cultures were held at 5 C for 24 hr prior to use, to allow maximum flavor development.

Preparation of cultured cream butters. Approximately 1,000 lb of fresh cream (28% fat) was pasteurized at 80 C for 30 min, cooled to 21.1, and inoculated with 1.5% of ripened bulk culture. The cream was incubated at 21.1 C for 1-2 hr, depending upon the activity of the culture and the amount of developed acidity

Received for publication July 25, 1966.

[1] Technical Paper no. 2130, Oregon Agricultural Experiment Station.

[2] Partially supported by funds granted by the American Dairy Association.

[3] Present address: International Flavors & Fragrances, Inc., 521 West 57th Street, New York.

sired. Following incubation, the cream was cooled to 4.4 C and held 14 hr prior to churning. The cream was churned at 10 to 13 C, depending upon the season of the year, and the butter granules were not washed. Salt content of the butters ranged from 0.5 to 1.5% and chilled buttermilk was used to adjust the moisture content during working. The finished butter was tempered at 4.4 C, printed into 1-lb blocks and samples taken for immediate analysis. Aliquots were wrapped and stored at −5 C for a maximum of 3 wk, until used for flavor panel evaluations.

Determination of diacetyl. The method of Elliker (8), with a few modifications, was used to determine diacetyl. The method was altered by using a 50-g sample and by collecting 50 ml of distillate into a 50-ml volumetric flask. A 10-ml aliquot of distillate was then reacted with 0.5 ml of concentrated hydrochloric acid and 0.1 ml of freshly prepared *o*-diaminobenzidine-HCl reagent. Absorbance was determined at 345 mμ instead of 400 mμ and the concentration of diacetyl taken from a standard curve. Recovery of added diacetyl from acidified milk and butter was 90% and all quantitative data were corrected on this basis.

Determination of short-chain acids. The acids in butter culture and cultured cream butter were determined by the procedure of Wiseman and Irvin (24). Samples of butter culture were analyzed intact. For butter, the acids were analyzed in the serum, isolated by warming butter to 40 C and centrifuging. Three grams of sample were acidified with 50% H_2SO_4 and mixed with sufficient dry silicic acid to give a free-flowing powder. This was added to the column as a cap and the column developed.

Since the acids were measured in the serum phase of butter, it was necessary to determine their distribution in the milk fat–serum binary phase system. The distributions of lactic, formic, and acetic acids between 80 g of milk fat and 17 ml of 6% aqueous NaCl solution held at 50 C were determined by adding known amounts of acids, shaking the mixture, and titrating the aqueous phase with standard base. The percentages of acids partitioned into the aqueous phase were lactic, 100; formic, 91; acetic, 86. These values were used to correct the amounts of acids found in the butter serum.

The pH of all products was determined with a Beckman Zeromatic instrument.

Determination of desirable levels of culture flavor compounds. The flavor panel data reported by Day et al. (5) was used as a guide for desirable levels of dimethyl sulfide in cultured cream butters.

For determination of a desirable level of diacetyl in cultured butter, samples were evaluated by an eight- to ten-member experienced flavor panel. One-pound butter samples were coded, tempered at 5 C, and evaluated in a room free of extraneous odors. A maximum of seven samples were displayed simultaneously for evaluation, and panel members could make comparisons. Over-all flavor desirability was scored on a seven-point hedonic ballot, with a scale ranging from 7, extremely desirable, to 1, extremely undesirable. One ballot was used by each judge for each sample and the data from three replications were averaged. Diacetyl in the samples was measured as previously described.

The data reported by Lindsay et al. (15) was used as a guide for desirable levels of acetaldehyde in relation to diacetyl content in butter cultures.

Preparation of synthetic culture flavor formulations. Initial formulations were prepared by adding aliquots of accurately prepared aqueous dilutions of individual culture flavor compounds to products. Flavor chemicals used were: Eastman-grade (Distillation Products Industries) diacetyl, acetaldehyde, and dimethyl sulfide; reagent-grade glacial acetic acid (Du Pont). Lactic acid (food grade—50%) was supplied by Monsanto Company. After basic formulations were established, complete culture flavor concentrates were prepared in propylene glycol carrier (Matheson Coleman and Bell).

Preparation of products for culture flavor formulation evaluations. Initial culture flavor formulations were evaluated in H_3PO_4-acidified raw whole milk heated for 1 hr in a boiling-water bath. The heated milk was acidified to pH 4.6 with 20% H_3PO_4 and the culture formulations added. These samples lacked desirable body and texture characteristics, and in later trials δ-gluconolactone (Fisher Scientific Company) was used as the acidogen (6), to obtain a more viscous product. However, use of δ-gluconolactone as the sole acidogen yielded products with an astringent flavor, similar to that described by Deane and Thomas (7). To overcome this defect in the latter stages of this investigation, the lactone was further purified by recrystallization from ethanol, and partially replaced by citric acid in the artificial buttermilk preparations.

Artificial buttermilk for large panel evaluations was prepared by adding citric acid (20 ml of 10% solution per kilogram) and δ-gluconolactone (8 g per kilogram) to whole milk heated in a boiling-water bath for 1 hr and cooled to

21 C. The samples were held without agitation for 12 hr at 21 C to develop a firm coagulum, then stored for 12 hr at 5 C to complete hydrolysis of the lactone (ca. pH 4.4). The acetic acid was then added (ca. pH 4.2) and the final pH was adjusted to 4.6 with $NaHCO_3$ (ca. 0.15%). Salt (0.15%) and the remainder of the flavor compounds were then added, to complete the preparations.

Sour cream was prepared from cream containing 18.5% fat and 2% added nonfat dry-milk solids was pasteurized at 80 C for 30 min and homogenized at 2,500 psi through the first stage of a Manton-Gaulin homogenizer. Citric acid (20 ml of 10% solution per kilogram) and recrystallized δ-gluconolactone (6 g per kilogram) were added to the cream (7), which was then held initially for 12 hr at 21 C and subsequently for 12 hr at 5 C to develop acidity (ca. pH 4.6). Flavor formulations were then added to the coagulated cream and mixed to complete the preparation. A good body and texture was obtained in this manner.

Cottage cheese curd (farmer style) was manufactured in the University creamery with a conventional long-set method, using fresh skim-milk and a mixed-strain cheese culture of commercial origin. The cream dressing was standardized to 11.5% milk fat and 22% total milk solids, and contained 0.3% stabilizer (Dariloid K). It was pasteurized at 80 C for 30 min and subsequently homogenized at 2,500 psi through the first stage of a Manton-Gaulin homogenizer. Culture flavor formulations and salt (1.1%) were added to the cream dressing, which was then combined with the curd (3:4). Finished creamed Cottage cheese was held 12 hr at 5 C before evaluation, to allow flavor equilibration (ca. pH 5.2).

For preparation of synthetic culture flavored butter, fresh cream (38% fat) was pasteurized at 80 C for 30 min, then churned in the previously described manner. Culture flavor formulations were added at the time of moisture adjustment, and finished butter samples obtained at the time of printing.

Flavor panel evaluations of culture flavor formulations. Initial culture flavor formulations and subsequent formulation modifications were evaluated by a small panel (eight to ten members) of experienced judges.

Samples of synthetic culture flavored buttermilk, butter, Cottage cheese, and sour cream, prepared as previously described, were presented to a preference panel composed of approximately 175 University students. The samples were scored on a nine-point hedonic scale from 9, like extremely, to 1, dislike extremely.

In preliminary preference panel trials, panel members expressed displeasure with eating butter and sour cream alone. A trial conducted to determine the influence of serving butter with a warm yeast roll showed insignificant differences between mean flavor scores of butter samples served with or without rolls. However, the absolute scores were higher when a roll was included. On this basis, butter samples were served with a roll and sour cream with a 2-oz bag of potato chips.

Butter samples were tempered to 4.4 C, cut into patties, then tempered at 10 C on the day prior to testing. The butter patties were served directly from the refrigerator on coded institutional-style butter-chip paper plates. A warm yeast roll, plastic knife, napkin, and ballot were included on each serving tray. Sour cream, buttermilk, and Cottage cheese samples were served in coded 3.5-oz paper cups.

Each serving tray for sour cream, Cottage cheese, and buttermilk contained two synthetically flavored samples—one high-quality natural product manufactured in the University creamery and one commercial sample obtained from a local supermarket. Cultured butter was presented in the same manner, except that no commercial sample was included.

TABLE 1

The pH, short-chain organic acid, and diacetyl content of butter cultures

Culture no.	Acetic acid[a]	Formic acid[a]	Lactic acid[a]	Diacetyl ($\times 10^{-4}$)[a]	pH
1	1.33	0.20	8.80	3.68	4.70
2	1.18	0.59	9.03	1.20	4.60
3	1.16	0.16	8.50	0.87	4.50
4	1.27	0.18	8.97	1.58	4.60
5	1.12	0.17	7.44	2.51	4.50
6	1.09	0.13	7.68	1.89	4.50
7	1.11	0.11	7.98	2.78	4.90
8	1.23	0.11	7.21	1.53	4.75
Average	1.22	0.17	7.99	2.01	4.64

[a] Grams/kilogram culture.

TABLE 2

Short-chain organic acid and diacetyl content of cultured butter and pH of corresponding butter serum

Butter no.	Acetic acid[a]	Formic acid[a]	Lactic acid[a]	Diacetyl ($\times 10^{-3}$)[a]	pH of butter serum
1	0.08	0.004	0.75	2.32	5.40
2	0.07	0.006	0.53	2.71	5.10
3	0.02	0.003	0.42	2.84	5.50
4	0.02	0.004	0.61	3.42	4.90
5	0.03	0.002	0.50	1.82	5.15
6	0.01	0.002	0.70	2.13	5.20
7	0.01	0.001	0.37	2.04	5.31
8	0.01	0.002	0.49	2.35	5.15
Average	0.03	0.003	0.54	2.45	5.21

[a] Grams/kilogram butter.

Results and Discussion

Results of analyses of eight high-quality butter cultures for acetic, formic, and lactic acids, diacetyl, and pH are shown in Table 1. Each culture was used in the manufacture of a lot of cultured cream butter and results of analyses of corresponding butter samples are shown in Table 2. Rather large variations in the concentrations of compounds and pH values of both butter cultures and butters are apparent. However, the average values obtained for the organic acids and pH determinations were useful in developing subsequent culture flavor formulations.

The diacetyl concentrations and average over-all flavor scores by the experienced panel for 14 butter samples are given in Table 3. Since judges directly compared all samples, the flavor scores tended to magnify differences. Previous reports (2, 22) have indicated that levels of 2.0 to 2.5 ppm of diacetyl in butter were too high and have undesirably harsh flavors. However, the butter samples evaluated in the present investigation were highly acceptable to the flavor judges. Apparently, the proper balance of other flavor compounds and individual flavor preferences are very important in determining desirable levels of diacetyl.

TABLE 3

Relationship of diacetyl content to over-all flavor score of cultured cream butter

Sample no.	Diacetyl	Average over-all flavor score[a]
	(ppm)	
1[b]	0.05	4.06
2	0.50	4.36
3	0.72	4.72
4	1.82	5.50
5	2.04	5.25
6	2.13	5.63
7	2.14	5.72
8	2.32	5.00
9	2.35	5.07
10	2.62	5.45
11	2.71	5.31
12	2.75	5.15
13	2.84	5.14
14	3.42	5.72

[a] From a seven-point hedonic scale where 7.0 = extremely desirable and 1.0 = extremely undesirable; 30 observations per sample.
[b] Sweet-cream butter.

Volatile esters have been long considered to contribute to the flavor of butter cultures and cultured cream butter (2, 11). In view of the presence of a large number of esters in culture distillates (16), this point was investigated further. The volatile ester contents of four high-quality butter cultures were determined by a spectrophotometric, hydroxamate procedure (14). Less than 1.0 ppm of esters expressed as ethyl acetate were found in the cultures. The flavor threshold value (21) of ethyl acetate was found to be approximately 10 ppm. Thus, the short-chain esters, such as ethyl formate and ethyl acetate, probably do not play a major role in culture flavor, and were not included in formulations. However, this observation does not preclude the possibility that low levels of some esters may contribute to the over-all flavor balance of high-quality butter cultures and cultured cream butters.

Earlier work in this laboratory (5) had shown that dimethyl sulfide could smooth out harsh diacetyl flavors. Another paper (15) reported that acetaldehyde contributed to the over-all full balanced flavor of butter cultures. The diacetyl-to-acetaldehyde ratio in full flavored cultures was found to be in the range of 4.4:1 to 3.2:1 and these data were used in initial culture flavor formulations.

On the basis of the foregoing data, culture flavor formulations were prepared and evaluated by trained judges. When a culture flavor formulation containing 2 ppm of diacetyl,

0.5 ppm of acetaldehyde, 1,250 ppm of acetic acid, and 25 ppb of dimethyl sulfide was added to acidified heated whole milk, it yielded a product that possessed an aroma very similar to natural butter cultures. The same formulation added to heated skimmilk was judged to have a fairly desirable culture aroma and flavor, but lacked the full flavor of the heated whole milk product. Artificial culture prepared from pasteurized skimmilk had the poorest flavor, and that prepared from homogenized milk was only slightly better. These results indicated that the added flavor compounds plus the flavor compounds present in heated whole milk were necessary for the typical aroma of high-quality butter cultures.

It was found also that the final pH of the medium exerted a strong influence on the overall culture flavor balance. Undesirable acetic acid-like flavors were observed in artificial butter cultures when the medium was below pH 4.5. However, when the pH was adjusted to the range of 4.6 to 4.8, a typical full aroma was obtained. Use of sodium bicarbonate to adjust the pH of these cultures introduced carbon dioxide and was found to markedly improve flavor. This observation is in agreement with the data reported by Andersen (1), which indicated that addition of 0.05 to 0.15% of sodium bicarbonate improved flavor of natural lactic cultures.

Trials were conducted to determine the effect of altering the amounts of flavor components in the artificial butter culture formulation. It was found that increasing dimethyl sulfide to 35 ppb caused a slight feedy flavor, whereas dropping the level to 15 ppb resulted in a slightly harsh diacetyl flavor. When acetaldehyde was added at a level of 0.75 to 1.0 ppm, a typically green flavor was observed. It was found that the acetaldehyde concentration could be lowered to about 0.2 ppm without seriously affecting the flavor and even appeared to improve the flavor if the diacetyl concentration was lowered to less than 1.0 ppm. However, if the diacetyl concentration was above 2.0 ppm, reduction of the acetaldehyde content caused a loss of full culture flavor and a somewhat harsh diacetyl flavor predominated. Altering the acetic acid level (± 100 ppm) did not noticeably influence the flavor. Formic acid was not added to the culture formulations, as the levels found in natural cultures were not considered important. Data by Morr et al. (20) indicated that comparable levels of formic acid were present in milk samples that had received heat treatments similar to those used in these studies.

Diacetyl was the predominant flavor note in the culture formulations. It was found that the intensity of culture flavor could be easily manipulated by simply changing the diacetyl concentration in the respective product flavor formulation. In general, levels of diacetyl from 0.5 to 1.00 ppm were found to give mild intensities, from 1.25 to 2.0 ppm intermediate intensities, and from 2.25 to 3.0 ppm pronounced intensities of culture flavor. With adjustments to account for differences in product manufacturing procedures, composition, and physical characteristics, culture flavor formulations were developed subsequently for butter, Cottage cheese, and sour cream. Recent work by Bennett et al. (3) has shown that fat, pH, and volatile acids affect the flavor perception of diacetyl. This is undoubtedly true for other flavor compounds, as well.

Flavor formulations that have given best results are presented in Table 4. Lactic acid was added only to butter, as the necessary acidity for buttermilk and sour cream was obtained from citric acid and δ-gluconolactone. Cottage cheese did not require added lactic acid, because sufficient acid was present in the curd to attain an adequate pH in the finished product. The data obtained for natural cultured butters (Table 2) showed the presence of an average of 540 ppm of lactic acid. However,

TABLE 4

Synthetic culture flavor formulations for dairy products

Compound	Cultured butter	Sour cream	Cottage cheese	Buttermilk[a]
	Concentration—mg/kg of product			
Lactic Acid	250.0	.....	.....	.....
Acetic Acid	30.0	30.0	30.0	1,250.0
Acetaldehyde	0.2	0.2	0.2	0.5
Dimethyl sulfide	0.08	0.025	0.025	0.025
Diacetyl (low level)	0.5	1.0	1.0	1.0
(high level)	2.0	2.0	2.0	2.0
pH of product	5.2	4.6	5.2	4.6

[a] Prepared from heated whole milk and containing ca. 0.15% of added sodium bicarbonate.

this amount of lactic acid added to sweet cream butter at the time of moisture adjustment caused the pH of the butter serum to drop to near 4.8. The resulting low pH yielded a harsh, unbalanced culture flavor in the butter. When the amount of lactic acid was reduced to 250 ppm, a desirable flavor was obtained and pH values from 5.1 to 5.4 were observed, depending on the buffer capacity of the serum. The cause of the discrepancy in measured lactic acid in natural cultured butters and that required for synthetically flavored butters is not clear. Some acid buffering system effective in cream and absent in churned butter may be involved.

It can be noted in Table 4 that the same culture flavor formulation was used for both sour cream and Cottage cheese. Some experienced judges objected to the flavor of the 25 ppb of dimethyl sulfide in Cottage cheese, but they did not object to its influence when the concentration was lowered to 20 ppb. Much higher concentrations of dimethyl sulfide were required in butter to give a fresh, full culture flavor than in the other products. Dimethyl sulfide is quite nonpolar, and naturally occurring dimethyl sulfide would be expected to be concentrated in the fat phase of milk or cream. Therefore, dimethyl sulfide is probably associated more with the fresh flavor of butter than with other cultured products. The acetic acid concentration of 30 ppm found as an average for cultured butters proved satisfactory for butter, Cottage cheese, and sour cream. In these products it was also found that a better flavor was obtained with 0.2 ppm of acetaldehyde than with the 0.5 ppm for buttermilk.

The experienced judges used in this study were in general agreement that the flavor formulations very closely simulated naturally produced high-quality butter culture flavor. Most found it difficult to distinguish between synthetically flavored and naturally cultured butter, Cottage cheese, or sour cream and all liked the smooth culture flavor produced by the formulations. The experienced judges did observe that the artificial buttermilk lacked the exact character of natural buttermilk. This was the most difficult product to work with and problems associated with tactile properties may have been at least partly responsible.

Samples of synthetic culture flavored products, containing the flavor formulations given in Table 4, were presented to large student preference panels shown to compare favorably with household consumer panels (4). To compare the synthetically flavored products with natural products, two synthetically flavored products, one high-quality natural product and one commercially obtained product, were included on each respective serving tray. A summary of the flavor panel evaluations is given in Table 5. No significant preference was found for either the synthetically flavored products or the high-quality natural products. The only samples that scored significantly lower than other samples in a group were the commercial sour cream and Cottage cheese.

It is of interest to note that no preference was shown for either the high or low level of diacetyl in the synthetically flavored products. Recent data reported by Hempenius et al. (13) showed that their consumer panel could not detect differences between creamed Cottage cheese samples unless the diacetyl content differed by 1.4 ppm or more. This suggests that considerable variations in diacetyl may not strongly influence consumer preferences.

The culture flavor concentrates used in the latter part of this work for flavoring butter, Cottage cheese, and sour cream were prepared in a propylene glycol carrier. The flavor compounds are very volatile and difficult to manip-

TABLE 5

Flavor preference scores for culture flavored dairy products

Sample identification	Cultured butter	Sour cream	Cottage cheese	Buttermilk
	Mean score[a]			
Commercial	[b]	5.25	6.02	3.72
OSU natural	6.38	5.73	6.22	3.69
Synthetic (low level diacetyl)	6.27	5.80	6.37	3.81
Synthetic (high level diacetyl)	6.29	5.75	6.41	3.71
LSD .01[c]	.34	.44	.37	.35
LSD .05	.26	.36	.28	.27

[a] Nine-point hedonic scale from 9, like extremely, to 1, dislike extremely; 175 observations per sample.
[b] Sample not available.
[c] Least significant difference at the .01 level.

ulate accurately, and extreme care is required in preparing formulations, to achieve desired results.

The potential use of the butter culture flavor concentrates in the dairy industry is undetermined at the present time. Recent emphasis on the development of methods for the manufacture of Cottage cheese (9, 23), sour cream, and buttermilk (7, 10, 12, 17) without the use of starter cultures has resulted in a need for a source of culture flavor in these products. In addition, there has been a long-standing need for a uniform desirable culture flavor in the butter industry. The culture flavor concentrates described in this paper were formulated on the basis of chemical analysis of naturally cultured products and subsequent flavor panel evaluations. The final flavor present in the synthetically flavored products results from a blending of those natural flavors present in milk-based products and those added by the culture flavor concentrates. The culture flavor concentrates will not mask defective flavors in a product.

Initial storage stability tests indicate that the flavor is reasonably stable. Work is continuing on this aspect, as well as on other related factors influencing the application of culture flavor concentrates.

References

(1) Andersen, V. B. 1961. Cultured Buttermilk and Sour Cream from Single-Strain Cultures of Lactis Streptococci. Milchwissenschaft, 16:128.

(2) Babel, F. J., and Hammer, B. W. 1944. Action of Butter Cultures in Butter: A Review. J. Dairy Sci., 27:79.

(3) Bennett, G., Liska, B. J., and Hempenius, W. L. 1965. Effect of Other Flavor Components on the Perception of Diacetyl in Fermented Dairy Products. J. Food Sci., 30:35.

(4) Calvin, L. D., and Sather, L. A. 1959. A Comparison of Student Preference Panels with a Household Consumer Panel. Food Technol., 13:469.

(5) Day, E. A., Lindsay, R. C., and Forss, D. A. 1965. Dimethyl Sulfide and the Flavor of Butter. J. Dairy Sci., 48:197.

(6) Deane, D. D., and Hammond, E. G. 1960. Coagulation of Milk for Cheese-Making by Ester Hydrolysis. J. Dairy Sci., 43:1421.

(7) Deane, D. D., and Thomas, W. R. 1964. Use of Chemical Compounds to Replace Lactic Cultures in the Manufacture of Sour Cream. J. Dairy Sci., 47:684.

(8) Elliker, P. R. 1945. Effect of Various Bacteria on Diacetyl Content and Flavor of Butter. J. Dairy Sci., 28:93.

(9) Ernstrom, C. A. 1965. Continuous Process Developed for Making Cottage Cheese Curd Without Culture. Canadian Dairy Ice Cream J., 44:32.

(10) Gerson, M. F., and McDermott, D. J. 1963. Direct Acidification Process Makes Cultured Dairy Products Without Culture. Food Process., 24(1):78.

(11) Hammer, B. W., and Babel, F. J. 1943. Bacteriology of Butter Cultures. A Review. J. Dairy Sci., 26:83.

(12) Hempenius, W. L., and Liska, B. J. 1964. Preliminary Observations on Production of Natural Flavor in Chemically Acidified Cream and Skimmilk. J. Dairy Sci., 47:1099.

(13) Hempenius, W. L., Liska, B. J., and Harrington, R. B. 1965. Consumer Preferences for Flavor in Creamed Cottage Cheese. J. Dairy Sci., 48:870.

(14) Lindsay, R. C. 1965. Flavor Chemistry of Butter Culture, Ph.D. thesis, Oregon State University, Corvallis.

(15) Lindsay, R. C., Day, E. A., and Sandine, W. E. 1965. Green Flavor Defect in Lactic Starter Cultures. J. Dairy Sci., 48:863.

(16) Lindsay, R. C., Day, E. A., and Sandine, W. E. 1965. Identification of Volatile Flavor Components of Butter Culture. J. Dairy Sci., 48:1566.

(17) Litchfield, J. H. 1964. Use Stabilizer and Acid to Replace Bacteria in Making Sour Cream, Buttermilk. Food Process., 25(5):130.

(18) Lundstedt, E., and Fogg, W. B. 1962. Citrated Whey Starters. II. Gradual Formation of Flavor and Aroma in Creamed Cottage Cheese After the Addition of Small Quantities of Citrated Cottage Cheese Whey Cultures of *Streptococcus diacetilactis*. J. Dairy Sci., 45:1327.

(19) Mather, D. W., and Babel, F. J. 1959. A Method for Standardizing the Diacetyl Content of Creamed Cottage Cheese. J. Dairy Sci., 42:1045.

(20) Morr, C. V., Harper, W. J., and Gould, I. A. 1957. Some Organic Acids in Raw and Heated Skimmilk. J. Dairy Sci., 40:964.

(21) Patton, S., and Josephson, D. V. 1957. A Method for Determining Significance of Volatile Flavor Compounds in Foods. Food Research, 22:316.

(22) Riel, R. R., and Gibson, C. A. 1961. The Use of Starter Distillate for Flavoring Butter. Food Technol., 15:137.

(23) Roahen, D. C. 1966. Mechanization of Cottage Cheese Manufacture. Am. Cottage Cheese Inst. Rev., 6(1):6.

(24) Wiseman, H. G., and Irvin, H. M. 1957. Determination of Organic Acids in Silage. J. Agr. Food Chem., 5:213.

Biochem. J. (1967) 104

The Incorporation of Acetate, Stearate and D(−)-β-Hydroxybutyrate into Milk Fat by the Isolated Perfused Mammary Gland of the Goat

By J. L. LINZELL, E. F. ANNISON, S. FAZAKERLEY and R. A. LENG
Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge, Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, and School of Rural Science, University of New England, Armidale, New South Wales, Australia

(*Received* 26 *September* 1966)
Biochem J. 104: (1), 34–42, (1967)

1. Mammary glands of lactating goats were perfused for 12·5–15hr. with heparinized whole blood and infused with a substrate mixture of glucose, acetate and amino acids (and sometimes chylomicra) containing either [1-^{14}C]acetate, D(−)-β-hydroxy[1-^{14}C]butyrate or [U-^{14}C]stearate. 2. There was a substantial net uptake of acetate by the glands and transfer of radioactivity into milk fat. Acetate was extensively utilized for the synthesis of milk fatty acids of chain length up to C_{14} and to a smaller extent for the synthesis of palmitate. 3. There was a small and variable net uptake of stearate and β-hydroxybutyrate and negligible oxidation of these substrates. However, tissue uptake was demonstrated by a substantial fall in specific radioactivity across the glands and an extensive transfer of radioactivity into milk fatty acids. 4. With β-hydroxybutyrate the labelling of milk fat was very similar to that with acetate, but the distribution of radioactivity suggested a cleavage into C_2 fragments of about 40%. 5. Labelled stearate gave rise to highly labelled stearate and oleate in the milk fat. Small amounts of radioactivity were detected in stearate of plasma triglycerides and oleate of plasma free fatty acids. 6. In experiments where there was a decline in milk-fat secretion late in perfusion, the milk fatty acids showed a marked decline in the proportion of stearate and oleate and a rise in the proportion of myristate and palmitate. This did not occur in experiments where milk-fat secretion was maintained at a higher level. 7. The present results confirm that there is a large pool of long-chain fatty acids in mammary tissue that can act as an endogenous source of these substrates.

Recently quantitative data on the role of glucose and fatty acids in mammary-gland metabolism in the undisturbed conscious goat have been obtained by combining arteriovenous difference studies with isotope dilution (Annison & Linzell, 1964; Annison, Linzell, Fazakerley & Nichols, 1967). These procedures allow mammary-gland metabolism to be correlated with overall metabolism, but the infusion of radioactive substrates into intact animals inevitably involves considerable dilution with body substrate pools, which may obscure precursor-product relationships in the gland. There are large net arteriovenous differences across the udder for acetate (Linzell, 1966; Annison & Linzell, 1964) and triglycerides (Barry, Bartley, Linzell & Robinson, [illegible]) and when these substrates are labelled and infused [illegible] milk fatty acids (Lyman, Frohman & Folley, 1964; Linzell, Hardwick, Linzell & Mepham, 1964). By contrast there is a negligible arteriovenous difference in FFA* in fed animals (Barry *et al.* 1963; Annison *et al.* 1967), and yet when labelled stearate, oleate or palmitate are infused a substantial amount of radioactivity is incorporated into the milk triglyceride fatty acids (Annison *et al.* 1967). Further analysis of this situation may be simplified by the use of the isolated perfused gland, where only one organ is involved and where the incorporation into milk of substrates of relatively high specific radioactivity can be studied for as long as 12–15hr. We have used this system to study the incorporation into milk fatty acids of [U-^{14}C]stearate, [1-^{14}C]acetate and [1-^{14}C]-β-hydroxybutyrate.

[illegible] mammary arteriovenous [illegible] of [illegible] (Shaw & [illegible]) [illegible] fatty acids [illegible] quoted.

1941) and goats (Barry *et al.* 1963). The ^{14}C-labelled compound is incorporated into milk casein and fatty acids (Kumar, Lakshmanan & Shaw, 1959; Palmquist, Davis & Brown, 1966), but some doubt exists as to whether or not the mechanism of incorporation into fatty acids is similar to that of butyrate, i.e. as two C_2 units (Lauryssens, Verbeke, Peeters & Reinards, 1960). Studies with DL-β-hydroxy[3-^{14}C]butyrate in the lactating cow (Luick & Kameoka, 1966) and in cell free systems of mammary tissue from lactating goats and rabbits (Kumar, Singh & Keren-Paz, 1965) suggested that the conversion of β-hydroxybutyrate into butyrate occurs largely without cleavage of the carbon chain. In the present work we have studied the incorporation of D(−)-β-hydroxy[1-^{14}C]butyrate into milk fatty acids because this is the naturally occurring isomer taken up by the goat's udder (Barry *et al.* 1963).

The amount of fat secreted by isolated perfused goat mammary glands is more variable than that of lactose and protein (Hardwick & Linzell, 1960), and less responsive to the availability of the precursors (acetate, triglycerides and β-hydroxybutyrate) that are taken from the plasma *in vivo* (Lascelles *et al.* 1964). Triglycerides and β-hydroxybutyrate are not usually necessary in the substrate mixture infused into isolated glands to maintain milk secretion, probably because the large pool of tissue triglycerides can serve as an endogenous supply of lipid substrate (Hardwick, Linzell & Mepham, 1963*a*). In some experiments after 8hr. of perfusion the secretion of milk fat falls, possibly owing to the exhaustion of endogenous substrates, but unfortunately this cannot be determined beforehand and may explain why the addition of triglycerides to the substrate mixture had little effect on the amount of fat secretion in two experiments performed by Lascelles *et al.* (1964). Another interest of the present experiments was therefore the assessment of the value of the isolated perfused lactating gland in studies of milk-fat secretion and synthesis, by comparing the uptake and incorporation of ^{14}C-labelled precursors into milk fat with that occurring *in vivo*.

MATERIALS AND METHODS

Experimental procedure. Isolated glands of lactating goats were perfused with heparinized whole blood for 12·5–15hr. according to the technique of Hardwick & Linzell (1960) and Hardwick, Linzell & Price (1961). A substrate solution of glucose, acetate and amino acids was infused continuously into the perfusate at a rate appropriate to the amount of milk being secreted. Milk was removed hourly, aided by an intra-arterial injection of oxytocin (40milliunits). Simultaneous arterial and venous blood samples were taken after 3–4hr. of perfusion and at hourly intervals thereafter. Blood flow was recorded every 10min.

In the experiment with acetate, both glands of one animal were perfused separately. Each gland received substrates containing [1-^{14}C]acetate in similar amounts but one gland received in addition goat chylomicra as a source of triglyceride (49ml., 3·1g. of fat infused in 9hr.). The chyle was collected 2 days before the experiment from a young goat with a cannula inserted in the main intestinal lymph duct by the method of Lascelles & Morris (1961).

The infusion of radioactive materials was started 2hr. after the start of the perfusion, and continued for the entire experiment with acetate, but only for 7 and 4·5hr. with stearate and β-hydroxybutyrate respectively. Stearate was bound to goat albumin as described by Annison *et al.* (1967).

Chemical methods. Analyses of glucose and acetate in the blood and artificial kidney fluid and of fat and lactose in the milk were carried out as reported by Hardwick & Linzell (1960). Analyses of plasma and milk fatty acids and of the concentration and specific radioactivity of blood CO_2 were carried out as described by Annison *et al.* (1967), except for the analyses on milk fats obtained during the [^{14}C]stearate and D(−)-β-hydroxy[^{14}C]butyrate infusions. Complete fatty acid analyses on these samples were made by temperature-programmed gas–liquid chromatography by using the method of Deman (1964).

Isolation and degradation of short-chain acids. Isolated milk fat was saponified with 5N-KOH, and the short-chain fatty acid fraction was separated by steam-distillation. Butyric acid was isolated by column chromatography on silica gel (Moyle, Baldwin & Scarisbrick, 1948) and degraded stepwise by the method of Pierce (1951), modified as reported by Leng & Annison (1963). Isolated $BaCO_3$ was assayed for specific radioactivity by suspending known weights of finely ground material in toluene containing low-density silica (38g./l.) and 2,5-diphenyloxazole (4g./l.) and the radioactivity measured in a liquid-scintillation counter (Packard Instrument Ltd. 4000 Series Tri-Carb).

Radioactive materials. [U-^{14}C]Stearate and [1-^{14}C]acetate were obtained from The Radiochemical Centre, Amersham, Bucks. D(−)-β-Hydroxy[1-^{14}C]butyrate was prepared by incubating [1-^{14}C]butyrate with sheep-liver slices, as described by Leng & Annison (1964): 82% of the radioactivity was in C-1 and 18% in C-3.

Calculations. (1) The hourly yield of fat secreted during perfusion is expressed as a percentage of the mean hourly yield before the experiment, determined by fat analysis of the total yield of milk for 3 days (hourly yield = daily yield/24).

(2) The R.Q. was calculated from the arteriovenous differences of O_2 and CO_2 in samples of arterial and venous blood taken simultaneously.

RESULTS

[1-^{14}C]*Acetate perfusion.* Two glands from one goat were perfused in separate systems for 15hr. Both glands received similar amounts of glucose, amino acids and acetate from the end of the second hour, together with [1-^{14}C]acetate at 0·6μc/min.; the left gland received in addition chylomicron lipid (3·1g. in 9hr.).

The quantities of milk fat obtained each hour, expressed as a percentage of the mean hourly yield for 3 days before the experiment, are shown in Fig. 1,

together with the time-course of the passage of radioactivity into the milk fat.

As in two previous perfusions (Lascelles *et al.* 1964) the differences in the amounts of milk fat secreted by the two glands cannot be confidently ascribed to the presence or absence of chylomicron lipid in the substrate mixture because such differences can occur in two glands of one animal treated identically (Hardwick & Linzell, 1960; Hardwick *et al.* 1961; J. L. Linzell, unpublished work). It is well known that after normal milking a small amount of milk, very rich in fat, is left in the udder. In perfusion experiments attempts are made to remove this residual milk fat by thorough milking with the aid of oxytocin at the start of perfusion, but it may be several hours before all this preformed milk fat is entirely cleared and variability in its removal makes it difficult to assess the true rate of fat secretion early in perfusion (e.g. see Fig. 1).

Other results were very similar for the two glands. The concentration of total acetate in the perfusates of both glands fluctuated over the range 0·7–1·4 mmoles/l. and there was a substantial uptake of acetate by both glands (7g. in 15hr., 1·6mg./100g. of tissue/min.), which was 70% of the mean value recorded by Linzell (1960) in conscious lactating goats. Throughout both experiments the R.Q. remained above 1 (mean ± S.E.M. 1·81 ± 0·37), which is characteristic of mammary glands actively synthesizing fatty acids (Folley, 1949). At the end of the perfusions there was still a substantial amount of total lipid in both glands, 3·1g./100g. in the gland that received chyle and 2·8g./100g. in the gland that did not, which supports the suggestion of Hardwick *et al.* (1963*a*) that mammary tissue has a large pool of fatty acids. The fatty acid composition and specific radioactivity of the tissue lipid were very similar to the milk fat, so that the tissue pool could have acted as an endogenous supply of substrate.

As in two similar perfusions reported by Hardwick, Linzell & Mepham (1963*b*), the magnitude of transfer of radioactivity from acetate to milk fat was considerable. The specific radioactivity of the milk fat produced by both glands rose steadily to a maximum after about 12hr. of perfusion, a value that was 64% of that of the acetate in the substrates, and between the tenth and eleventh hours of perfusion the total radioactivity recovered in the milk fat was 54% of the radioactivity being infused each hour (Fig. 1). The labelling of individual higher fatty acids (Table 1) was in agreement with the results obtained in the whole animal (Popjak, French, Hunter & Martin, 1951; Anderson *et al.* 1957). In spite of the high radioactivity of the infused acetate, there was no detectable incorporation of radioactivity into fatty acids of chain length C_{17} and higher. In the final stages of the infusion the specific radioactivities of laurate, myristate and palmitate were roughly similar (Table 1).

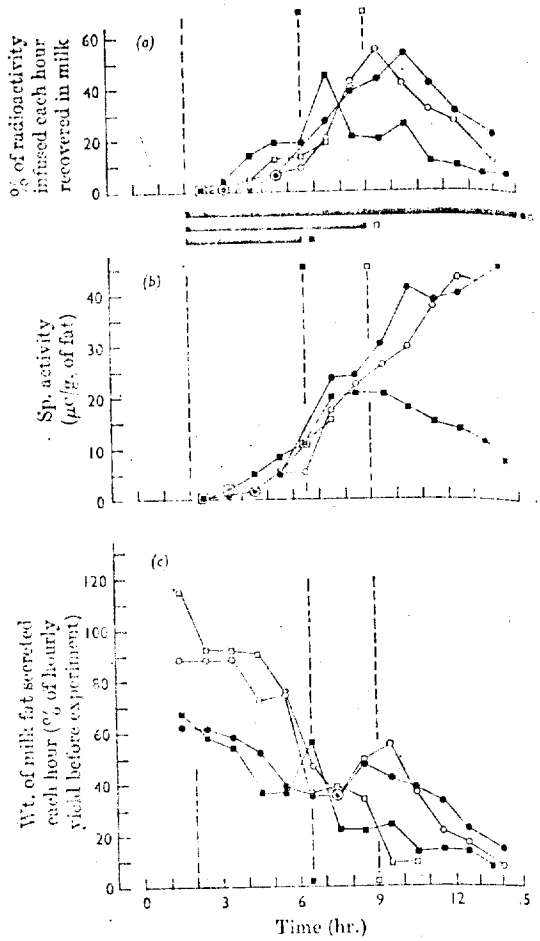


Fig. 1. Time-course of milk-fat secretion and incorporation of radioactivity into milk fat during perfusion of four goat mammary glands. A substrate mixture of glucose, acetate and amino acids was infused continuously. After 2hr. the infusion of labelled substrate was started. One gland received D(−)-β-hydroxy[1-^{14}C]butyrate (▲) for 15hr., another [U-^{14}C]stearate (□) for 7hr. and the two glands of a third goat [1-^{14}C]acetate for 13hr. with (●) or without (○) chylomicra in the substrate mixture.

The changes in fatty acid composition and changes in the quantities of the major components secreted in the milk each hour are shown for the gland receiving chyle in Table 1 and Fig. 2. The fatty acid composition of the milk of the gland not receiving chyle was very similar. The [illegible] stearate was fed as well as [illegible] than that of the other major fatty acids, [illegible]

Table 1. *Composition and specific radioactivities of the higher fatty acids of milk secreted during the perfusion of an isolated mammary gland with* [1-^{14}C]*acetate*

The substrate mixture contained [1-^{14}C]acetate (67μc/g.) infused at about 0·5g./hr. and chylomicron lipid at 0·5g./hr. falling to 0·25g./hr. The fatty acid nomenclature in this and subsequent Tables is that of Farkquhar, Insull, Rosen, Stoffel & Ahrens (1959).

	Sample (time of perfusion)									
	3hr.		6hr.		8hr.		12hr.		Tissue lipid	
Fatty acid	Composition (%)	Sp. activity (μc/g.)	Composition (%)	Sp. activity (μc/g.)	Composition (%)	Sp. activity (μc/g.)	Composition (%)	Sp. activity (μc/g.)	Composition (%)	Sp. activity (μc/g.)
12:0	3·7	0·7	3·5	53·0	5·2	27·0	8·2	35·2	2·9	33·2
12:br	—	—	—	—	1·9	—	4·0	—	0·6	—
14:1	—	—	—	—	0·2	—	0·4	—	0·2	—
14:0	10·2	0·3	11·0	24·8	12·7	30·4	8·7	36·9	9·5	39·2
14:br	0·7	—	0·6	—	1·2	—	0·8	—	—	—
15:0	0·8	—	0·7	—	0·5	—	0·8	—	0·4	—
16:0	28·8	0·1	28·8	30·2	29·7	29·4	34·6	34·1	33·5	28·8
16:1	1·9	—	1·9	5·4	1·3	5·4	2·5	—	1·6	—
16:br	0·7	—	1·4	—	3·5	—	0·4	—	1·0	—
17:0	0·5	—	0·4	—	0·5	—	—	—	0·5	—
18:0	16·4	—	12·4	—	10·4	0·2	10·2	—	12·1	1·0
18:1	31·1	—	36·7	—	27·3	0·7	29·4	—	35·5	0·1
18:2	2·3	—	2·7	—	1·2	—	—	—	1·7	—

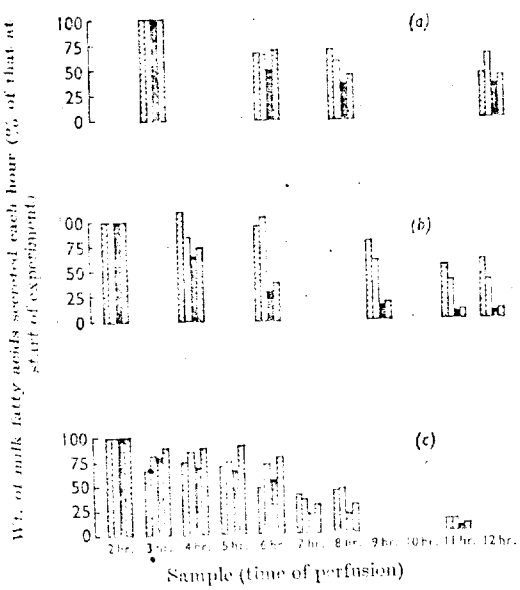


Fig. 2. Quantities of milk fatty acids secreted each hour in [illegible] perfusions, expressed as percentages of the quantities [illegible] at the start of perfusion, i.e. after a [illegible] period fat was [illegible]. (*a*) Perfusion with [1-^{14}C]acetate, with the [illegible] being given chylomicron lipid (Table 1). Perfusion [illegible] glands given acetate but no source of long-chain fatty acid. (*b*) with carrier-free (+)-3β-hydroxy[1-^{14}C]butyrate; (*c*) with [U-^{14}C]stearate. ▥, Myristate; ▤, palmitate; ▦, stearate; ▩, oleate.

Table 2. *Fatty acid composition and specific radioactivities of plasma free fatty acids in perfusate arriving at* (A) *and leaving* (V) *the perfused mammary gland after* 6·5*hr. of* [*U*-^{14}C]*stearate infusion*

	Composition (%)		Sp. activity (μc/g.)	
Fatty acid	A	V	A	V
12:0	2·2	6·1	—	—
14:0	5·7	7·0	—	—
15:0	1·7	1·0	—	—
16:0	31·6	31·7	—	—
16:1	10·4	7·4	—	—
17:0	3·8	2·9	—	—
18:0	17·1	14·0	2795	1145
18:1	25·1	24·9	263	209
18:2	2·8	1·7	—	—

the secretion of palmitate had fallen much less than that of myristate, stearate and oleate.

[*U*-^{14}C]*Stearate perfusion.* The labelled fatty acid was bound to goat albumin as described by Annison *et al.* (1967) and infused without added carrier into the perfusate at 0·5μc/min. for 7hr. from the second hour after the start of the perfusion, in an experiment lasting 13hr. Chylomicron fat was not included in the substrates. Plasma FFA concentrations remained low, but constant (0·15–0·16m-mole/l.), throughout the perfusion. There was a very small arteriovenous difference of FFA

Table 3. *Fatty acid composition and specific radioactivities of milk fat secreted by an isolated perfused goat mammary gland during the infusion of [U-^{14}C]stearate (32 μc/hr.) for 7 hr. from the start of the second hour*

Fatty acid	Sample (time of perfusion) 2hr. Composition (%)	3hr. Composition (%)	4hr. Composition (%)	4hr. Sp. activity (μc/g.)	5hr. Composition (%)	5hr. Sp. activity (μc/g.)	6hr. Composition (%)	6hr. Sp. activity (μc/g.)	7hr. Composition (%)	7hr. Sp. activity (μc/g.)	8hr. Composition (%)	8hr. Sp. activity (μc/g.)	11hr. Composition (%)
4:0	1·8	1·6	1·6	—	1·7	—	1·5	—	1·4	—	0·9	—	0·9
6:0	2·1	2·2	1·9	—	1·9	—	2·0	—	2·2	—	2·0	—	1·8
8:0	2·5	2·5	2·5	—	2·3	—	2·3	—	2·5	—	2·5	—	2·7
10:0	8·6	7·1	7·5	—	8·0	—	8·0	—	9·8	—	8·7	—	9·6
?	0·2	0·2	0·2	—	0·3	—	0·3	—	—	—	—	—	—
12:0	3·7	3·5	3·6	—	3·8	—	4·3	—	5·2	—	5·0	—	5·6
14:0	9·0	7·5	8·4	—	8·1	—	6·5	—	11·5	—	12·5	—	14·2
14:br	0·5	0·5	0·4	—	0·4	—	0·5	—	0·5	—	—	—	—
15:0	0·6	0·5	0·5	—	0·5	—	0·6	—	0·5	—	—	—	—
15:br/1	0·5	0·8	0·7	—	0·8	—	0·7	—	0·7	—	0·7	—	0·8
15:br/2	0·3	0·5	0·4	—	0·2	—	0·3	—	0·3	—	—	—	0·3
16:0	22·1	22·7	23·5	—	21·3	—	24·1	—	24·6	—	30·9	—	33·6
16:1	1·8	1·8	1·9	—	2·1	—	1·8	—	1·9	—	2·1	—	2·0
16:br	0·9	0·8	0·8	—	0·8	—	0·9	—	0·8	—	0·9	—	0·8
17:0	0·8	0·7	0·7	—	0·7	—	0·6	—	0·6	—	0·5	—	0·5
?	0·5	0·4	0·5	—	0·4	—	0·4	—	0·4	—	0·4	—	0·4
18:0	13·0	12·8	11·2	3·8	10·9	6·4	10·9	17·6	8·5	105	7·5	126	4·9
18:1	26·3	29·4	29·2	1·3	30·9	4·3	31·6	14·6	25·4	41·5	24·2	67	20·3
18:2	2·7	2·6	2·7	—	3·0	—	3·0	—	2·6	—	3·0	—	1·6
>18:0	2·1	1·9	1·8	—	1·9	—	1·5	—	0·6	—	—	—	—

Table 4. *Fatty acid composition and specific radioactivities of milk fat secreted by the isolated perfused goat mammary gland during the infusion of* D(−)-*β-hydroxy*[1-^{14}C]*butyrate* (20 μc/hr.) *for* 4·5 *hr. from the start of the second hour*

Fatty acid	Sample (time of perfusion) 2hr. Composition (%)	2hr. Sp. activity (μc/g.)	4hr. Composition (%)	4hr. Sp. activity (μc/g.)	6hr. Composition (%)	6hr. Sp. activity (μc/g.)	9hr. Composition (%)	9hr. Sp. activity (μc/g.)	11hr. Composition (%)	12hr. Composition (%)	12hr. Sp. activity (μc/g.)
4:0	1·8	—	1·9	—	1·7	61·6	1·4	—	1·3	1·2	—
6:0	1·9	—	2·1	—	2·2	51·7	1·8	—	1·9	1·5	—
8:0	2·3	—	2·5	—	2·7	65·6	2·3	—	2·1	1·9	—
10:0	5·2	13·6	7·3	34·7	9·6	55·8	9·8	42·1	9·7	9·7	31·8
?	0·1	—	0·1	—	0·1	—	0·1	—	0·1	0·2	—
12:0	2·2	9·9	3·1	40·9	4·3	64·8	5·8	51·4	5·9	6·4	13·6
14:0	4·3	11·3	5·6	33·5	7·7	51·4	10·6	32·7	12·0	12·5	15·6
14:br	0·2	—	0·3	—	0·2	—	0·1	—	0·1	0·4	—
15:0	0·4	—	0·4	—	0·3	—	0·2	—	0·2	0·4	—
15:br/1	0·6	—	0·5	—	0·4	—	0·3	—	0·2	0·5	—
15:br/2	0·3	—	0·2	—	0·2	—	0·2	—	0·2	0·2	—
16:0	18·1	2·6	19·4	10·0	28·3	35·2	33·9	27·0	36·1	37·5	16·4
16:1	2·7	—	2·1	—	1·8	—	2·1	—	1·9	1·5	—
16:br	1·0	—	0·9	—	0·6	—	0·6	—	0·7	0·7	—
17:0	0·9	—	0·7	—	0·5	—	0·5	—	0·4	0·4	—
?	0·7	—	0·6	—	0·4	—	0·4	—	0·3	0·3	—
18:0	15·0	—	12·0	—	7·9	—	6·5	—	5·5	4·8	—
18:1	37·2	—	34·8	—	26·6	—	20·1	—	17·8	17·4	—
18:2	3·9	—	4·0	—	3·0	—	2·9	—	3·1	1·6	—
>18:0	1·2	—	1·5	—	1·5	—	0·4	—	0·5	0·9	—

across the mammary gland, and the composition of the FFA fraction showed only slight changes (Table 2). As in the whole animal, however, there was a marked decline in the specific radioactivity of stearate across the gland, indicating substantial uptake of FFA accompanied by FFA production by the gland. The uptake of FFA was confirmed by the extensive transfer of radioactivity into milk fat (Fig. 1), radioactivity being found in C_{18} fatty acids only.

Comparison of the specific radioactivities of arterial and venous blood carbon dioxide showed that the carbon dioxide produced by the gland (0·47 μc/g. of C) was very low relative to that of circulating [U-^{14}C]stearate (Table 2), indicating that this acid made only a small contribution to oxidative metabolism in the isolated perfused mammary gland. The R.Q. was 1·20 ± 0·15.

Labelled oleate appeared both in plasma FFA and in milk fat (Tables 2 and 3). Some radioactivity appeared in arterial and venous triglycerides, but the concentrations of this fraction were variable, and in some cases venous concentrations were higher than arterial, suggesting triglyceride production by the gland. There was negligible labelling of phospholipids, but the sterol ester fraction showed slight radioactivity (3 μc/g.).

The amount of milk fat obtained each hour was high in the first 6hr. of the perfusion, but then declined to about 50% of the pre-perfusion level during the last 3hr. Milk fatty acid composition showed marked changes (Table 3 and Fig. 2). There was a steady fall in the secretion of stearate and a fall in oleate after 6hr., so that after the seventh hour the proportions of myristate and palmitate in the total milk fatty acids had appreciably increased. At the end of perfusion the tissue contained 1·73g. of lipid/100g. The milk fat of highest specific radioactivity was fractionated to isolate the small amount of FFA. On analysis this was shown to be similar in composition and radioactivity to the fatty acids of the triglyceride fraction.

D(−)-*β-Hydroxy*[1-^{14}C]*butyrate perfusion*. The labelled material, essentially carrier-free, was infused into the perfusate at 0·26 μc/min. for 4·5hr. of a perfusion lasting 12·5hr. Blood concentrations remained at 0·6–0·8mg./100ml. throughout the perfusion, and arteriovenous differences were small and variable. Substantial uptake of β-hydroxybutyrate was shown by the fall in specific radioactivity across the mammary gland (660 to 544 μc/g. of C), and the extensive transfer of radioactivity into milk (Fig. 1). A comparison of the specific radioactivity of the carbon dioxide produced by the gland (from arterial and venous values) showed that only 1·4% of the carbon dioxide originated from the circulating β-hydroxybutyrate. The R.Q. was 2·67 ± 0·57.

During the perfusion the hourly milk yield was 50–60% of the mean hourly yield on the goat 3 days before the experiment, but the fat content fell sharply (Fig. 1). This was accompanied by a steady fall in the proportions of stearate and oleate, and compensatory increases in the proportions of palmitate, and acids of chain length C_{14}, C_{12} and C_{10}, but not of chain length C_8, C_6 and C_4 (Table 4 and Fig. 2). At the end of perfusion the tissue contained only 0·45g. of lipid/100g. The specific radioactivity of milk fat reached a maximum after 6–7hr. (Fig. 1), and the specific radioactivities of the short-chain and long-chain fatty acids of this sample were determined. Roughly similar values were obtained for each acid in the series butyrate–myristate, with palmitate about 30% lower. The acids of carbon chain length C_4, C_8 and C_{12} were somewhat higher in specific radioactivity than C_6, C_{10} and C_{14} acids, which might suggest that β-hydroxybutyrate was incorporated into milk fatty acids to some extent as a C_4 unit. However, the distribution of radioactivity in the carbon chain of milk butyrate was 69% in C-1 and 31% in C-3, which must be compared with that of the labelled precursor, D(−)-β-hydroxybutyrate (82% in C-1 and 18% in C-3). These results are consistent with 41% cleavage of β-hydroxybutyrate to the C_2 unit before conversion into butyrate.

DISCUSSION

The present results show that the isolated perfused mammary gland can give valuable data on milk-fat synthesis, in spite of the fact that it takes several milkings to remove completely preformed milk fat, and it is not as immediately responsive to variations in the availability of lipid precursors as it is to precursors of lactose and protein.

The pattern of incorporation of labelled acetate and stearate into milk fat by the isolated perfused mammary gland was closely similar to that occurring *in vivo*, but the specific radioactivity of the materials in the perfusate was roughly tenfold greater than that achieved in the whole animal (Annison *et al.* 1967). This gave rise to highly labelled milk fat, and examination of the constituent fatty acids has confirmed conclusions reached in earlier work on the role of acetate and stearate in milk-fat synthesis. Acetate was extensively used for the synthesis of fatty acids of chain length C_6–C_{14}, and contributed to a smaller extent to palmitate production. Negligible synthesis of acids of greater chain length was observed from acetate. Stearate was readily taken up by the mammary gland, transferred to milk fatty acids and gave rise to large amounts of oleate, some of which appeared in the milk fatty acids and in the blood leaving the gland in the plasma FFA.

The isotopic data support the hypothesis that, in ruminants, milk short-chain fatty acids are derived from plasma acetate and β-hydroxybutyrate and the long-chain ones from plasma long-chain fatty acids. The present experiments add more weight by showing that these processes are to some extent independent. In two of the four experiments the quantity of milk fat secreted each hour was well maintained for 12hr. and in these the milk fatty acid composition showed little change. The other two glands were secreting less than half the quantity of milk fat late in perfusion, and in these experiments, in which lipid was not included in the substrate mixture, there was a steady decline to very low rates in the hourly secretion of stearate and oleate in milk fat, whereas the secretion of myristate and palmitate (largely formed from acetate, which was supplied continuously) was well maintained for 8hr. and then declined more slowly than that of stearate and oleate (Fig. 2). In these experiments less fat was present in the tissue at the end of perfusion, and this confirms that lactating mammary glands have a large pool of long-chain fatty acids (Hardwick *et al.* 1963*a*), which can act as an endogenous source for the synthesis of milk fat, in those experiments where lipid is not included in the substrate. It is noteworthy that the fatty acid composition of mammary-tissue lipid is remarkably similar to that of milk fat (Table 1).

The magnitude of the mammary-gland fatty acid pool is probably responsible for the slow transit of radioactivity into milk fat, as it is *in vivo* (Hardwick *et al.* 1963*a*). Evidence for this is that the residual tissue lipid is low in those experiments where there is a marked fall in milk-fat secretion late in perfusion, suggesting that the tissue fatty acid pool was being used as a source of endogenous substrates. It is significant that, in such cases, the proportion of stearate and oleate in the milk fatty acids falls markedly, because these are the milk fatty acids that are believed to be incorporated from plasma long-chain precursors and the isotopic evidence is clear that mammary tissue cannot form these fatty acids from acetate.

The metabolism of β-hydroxybutyrate has not been previously studied with isotopes in the goat and in only one other study has the naturally occurring isomer been used in a lactating animal (Palmquist *et al.* 1966). In our experiment negligible amounts of the D(−)-β-hydroxy[1-^{14}C]butyrate were oxidized, but in spite of low circulating plasma concentrations, which were only 10–20% of that in the normal animal (Barry *et al.* 1963), a large proportion of the radioactivity infused was transferred to milk fat. The pattern of labelling in the milk fatty acids was very similar to that with acetate, but suggested that incorporation proceeds by at least two routes. The distribution of radioactivity in butyrate isolated from milk fat indicated about 40% cleavage to C_2 units, in contrast with the findings of Kumar *et al.* (1965) that conversion of β-hydroxybutyrate into butyrate proceeds without cleavage. The specific radioactivities of the lower fatty acids also suggested that some cleavage of β-hydroxybutyrate must have occurred in the perfused gland (Table 4), but the slightly higher specific radioactivity of C_4, C_8 and C_{12} fatty acids than of C_6, C_{10} and C_{14} fatty acids might suggest some incorporation of C_1 units. The lactating mammary gland is not usually regarded as a site of ketone-body production, since there is normally a positive arteriovenous difference of β-hydroxybutyrate and none of acetoacetate (Barry *et al.* 1963), but the relatively unchanged concentration of β-hydroxybutyrate in the perfusate indicated continuous production in our experiment. Kronfeld & Kleiber (1959) have suggested and Kronfeld, Raggi & Ramberg (1966) demonstrated that acetoacetate is produced by the udder of the ketotic cow *in vivo*.

The present experiments clearly demonstrate the extensive ability of the goat mammary gland to form oleate from stearate. At maximum the specific radioactivity of the milk oleate was above half that of the milk stearate and in all experiments the fall in oleate secretion was less than the fall in stearate secretion.

We are grateful to Mr I. R. Fleet, Mr B. D. Burton and Miss A. Boyd for skilful technical assistance.

REFERENCES

Annison, E. F. & Linzell, J. L. (1964). *J. Physiol.* **175**, 372.

Annison, E. F., Linzell, J. L., Fazakerley, S. & Nichols, B. W. (1967). *Biochem. J.* **102**, 637.

Barry, J. M., Bartley, W., Linzell, J. L. & Robinson, D. S. (1963). *Biochem. J.* **89**, 6.

DeMan, J. M. (1964). *J. Dairy Sci.* **47**, 546.

Farquhar, J. W., Insull, W., Rosen, P., Stoffel, W. & Ahrens, E. H. (1959). *Nutr. Rev.* **17** (Suppl.), 18.

Folley, S. J. (1949). *Biol. Rev.* **24**, 316.

Hardwick, D. C. & Linzell, J. L. (1960). *J. Physiol.* **154**, 547.

Hardwick, D. C., Linzell, J. L. & Mepham, T. B. (1963*a*). *Biochem. J.* **87**, 4P.

Hardwick, D. C., Linzell, J. L. & Mepham, T. B. (1963*b*). *Biochem. J.* **88**, 213.

Hardwick, D. C., Linzell, J. L. & Price, S. M. (1961). *Biochem. J.* **80**, 37.

Kronfeld, D. S. & Kleiber, M. (1959). *J. appl. Physiol.* **14**, 1033.

Kronfeld, D. S., Raggi, F. & Ramberg, C. F. (1966). *J. Dairy Sci.* **49**, 733.

Kumar, S., Lakshmanan, S. & Shaw, J. C. (1959). *J. biol. Chem.* **234**, 754.

Kumar, S., Singh, V. N. & Keren-Paz, R. (1965). *Biochim. biophys. Acta*, **98**, 221.

Laseelles, A. K., Hardwick, D. C., Linzell, J. L. & Mepham, T. B. (1964). *Biochem. J.* **92**, 36.

Laseelles, A. K. & Morris, B. (1961). *Quart. J. exp. Physiol.* **46**, 199.

Lauryssens, M., Verbeke, R., Peeters, G. & Reinards, M. T. (1960). *J. Dairy Res.* **27**, 151.

Leng, R. A. & Annison, E. F. (1963). *Biochem. J.* **86**, 319.

Leng, R. A. & Annison, E. F. (1964). *Biochem. J.* **90**, 164.

Linzell, J. L. (1960). *J. Physiol.* **153**, 492.

Ludeke, J. R. & Kameoka, K. K. (1966). *J. Dairy Sci.* **49**, 98.

Moyle, V., Baldwin, R. & Starisbrieb, R. (1948). *Biochem. J.* **43**, 308.

Palmquist, D. L., Davis, C. L. & Brown, R. E. (1966). *J. Dairy Sci.* **49**, 738.

Phares, E. F. (1951). *Arch. Biochem. Biophys.* **33**, 173.

Popják, G., French, T. H. & Folley, S. J. (1951). *Biochem. J.* **48**, 411.

Popják, G., French, T. H., Hunter, G. D. & Martin, A. J. P. (1951). *Biochem. J.* **48**, 612.

Shaw, J. C. & Knoch, C. B. (1941). *J. biol. Chem.* **138**, 227.

BIOASSAY OF DIURETICS

WERNER L. LIPSCHITZ, ZARCH HADIDIAN AND ANDREW KERPCSAR

From the Lederle Laboratories, Pearl River, N. Y.

Received for publication April 25, 1942

J. Pharm. 79:97-110 (1943)

Although many studies have been made of the mechanism of diuresis, there has been no systematic quantitative study done on diuretics. Such an investigation should include a determination of the dose-action relationship of various diuretics by a reliable method, and an estimation of their relative potency with reference to a suitable standard. To be of practical use in future studies, the method should be simple, accurate and applicable to a variety of substances. A method using rats for the estimation of anti-diuretic potency has been described by Burn (1). This method, or a slight modification of it, has been used for diuretic assays by Marx (2), Herte (3), Jaretzky and Neuwald (4), and Vollmer (5). Controversial results reported in the last two citations clearly demonstrate the inadequacy for this purpose of the anti-diuretic assay method, which is devised for the measurement of a decrease in urinary excretion and requires a normally high excretion of urine. For the measurement of an increase, it is desirable to have the normal urinary excretion at a minimum.

In this paper a method suitable for diuretic assays will be described, and some results of its application to the study of several diuretics, most of which are in common use, will be presented.

METHOD. The procedure to be described for the determination of diuretic potency of drugs differs from Burn's in four important points: (1) the amount of liquid fed is reduced to half of that used in the anti-diuretic method, i.e., 25 cc./kgm. instead of 50 cc./kgm.; (2) isotonic sodium chloride solution rather than tap water is used for feeding; (3) the number of rats used for each dose is increased from four to eight; and finally (4) the total volume of excretion during a definite period of time, rather than the time necessary for the rate of excretion to reach a maximum, is taken as the measured variable. As a result of these modifications, basal urinary excretion is reduced to a minimum, and variations among groups are reduced considerably.

Healthy male Sherman rats weighing 140 to 240 grams were fasted for eighteen hours previous to the experiment, and were then given the substance to be tested in proportion to their weight. When administered orally, the substance was dissolved or suspended in isotonic sodium chloride solution at approximately body temperature, and fed to the animals through a stomach tube. After the feeding, the animals were placed in sub-groups of four in special metabolism-cages (Fig. 1), and remained there without food or water for the duration of the experiment. Two sub-groups were used for each dose. At the end of a definite period of time, the animals were taken out of the cages and the total volume of urine excreted by each group was measured. For all of the substances investigated, except bismuth potassium tartrate and salyrgan, a period of five hours was found to be adequate, as indicated by a great decrease in the rate of excretion during the fifth hour. Since, in the process of feeding, the animals lose all their urine, it was considered important at the end of the experiment to expel the urine from the bladder by pulling at the base of the tail.

In most experiments five groups of approximately equal weight were used. One group was kept as a control and was fed normal saline solution. In most cases two groups were fed suitable

doses of urea, and the remaining groups were given varying doses of the substance to be tested. The volume of urine excreted during five hours was in each case expressed as per cent of the liquid administered.

This percentage gives a measure of *urinary excretion* independent of group weight, and may be used in the calculation of *diuretic action*. The ratio $\frac{\text{Urinary excretion in test group}}{\text{Urinary excretion in control}}$ could be used as a measure of diuretic action for a given dose. In practice, however, it was found necessary to compare test groups receiving a new preparation with parallel test groups receiving a standard drug. This reduces the variability materially. Since the same control group appears in the estimation of relative potency only as a constant term which would cancel out, it is simpler to use the percentage of urinary excretion for such estimates. The control then serves as a check on experimental conditions as in the assay of Vitamin A, but does not enter into the calculation of the relative potency. *Diuretic activity* is the diuretic potency of a substance referred to that of the standard, urea, the activity of which is taken equal to one. Urea was chosen as the standard diuretic because

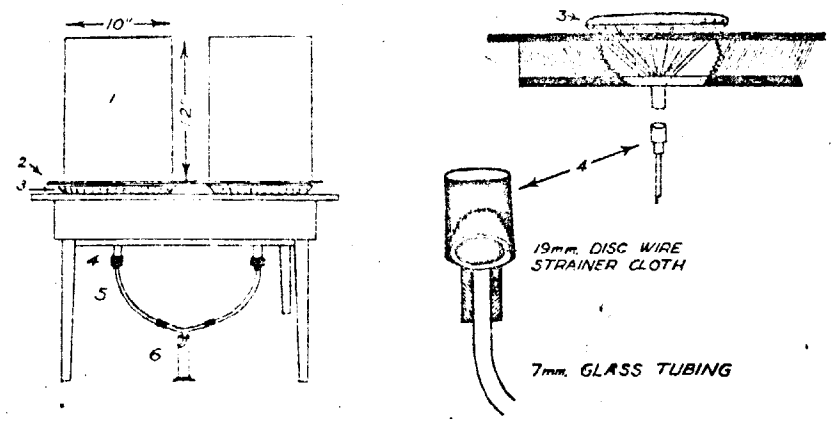


FIG. 1. SKETCH OF THE METABOLISM CAGE

(1) Glass cylinders, 10 inches in diameter and 12 inches high; (2) circular wire screen; (3) rib funnel - 1 gallon capacity; (4) vaccine stopper with fine screen inset; (5) glass tubing with "Y" joint; (6) volumetric cylinder.

it is stable *in vivo* as well as *in vitro*, a relatively weak diuretic and nontoxic in very high doses.

Bismuth potassium tartrate was injected intraperitoneally in doses varying from 2.5 to 50 mg./kg., and the proper amount of normal saline was administered orally following the injections. Salyrgan was injected in doses varying from 6 to 30 mg./kg.; and then normal saline or 1% ammonium chloride in saline (4.7 mM/kg.) was fed. Ammonium chloride was chosen for its well known potentiation of salyrgan diuresis (6). When it was used, determinations of diuretic action were made with reference to the urinary excretion of the group receiving ammonium chloride alone. With these two substances it was found necessary to keep the animals on test for approximately twenty-four hours.

In each instance several experiments were done with three or four different doses of the substance to determine the type of dose-action curve to be obtained. Within a fairly large range of doses, most of the substances yielded an almost linear relationship between log dose and log effect. In several instances the curve was approximately linear only within the range of very low doses. The best fitting straight line for each set of data was calculated from the regression equation (1)

$$y = \bar{y} + b(X - \bar{X}) \qquad (1)$$

where $\bar{y}$ is the mean value of y (log effect), $\bar{x}$ the mean value of x (log dose) and b the regression coefficient determined from the equation

$$b = \frac{\Sigma y(x - \bar{x})}{\Sigma(x - \bar{x})^2} \quad \text{(II)}$$

In subsequent experiments the effect of two or more doses of the substance was compared with that of one of more doses of urea. The dose-action curves of the substance and of urea

TABLE 1

Variability of urinary excretion among groups on same day

Control rats, fed 25 cc./kgm. of 0.9% NaCl solution

RATS PER GROUP	EXPER. DATE	NUMBER OF GROUPS	URINARY EXCRETION: $\frac{\text{VOL. EXCRETED IN 5 HOURS}}{\text{VOL. ADMINISTERED}} \times 100$ Original data								Mean
	1941										
4	9-19	4	34.3	23.8	32.3	32.4					30.7
	9-30	8	21.5	32.6	30.5	40.2	16.8	33.9	19.6	24.2	27.4
	10-8	8	13.8	12.0	14.4	22.4	19.3	7.2	14.3	41.1	18.1
	67 more exper.	2									
Average											32.7
8	12-29	2	23.0	29.7							26.4
	12-31	5	30.4	23.3	43.5	40.8	41.8				36.0
	1942										
	1-5	5	37.2	22.3	32.0	35.0	36.1				32.5
	1-7	3	50.5	52.1	47.2						49.9
	1-14	2	33.4	29.6							31.5
	1-15	3	33.1	35.4	28.3						32.3
Average											31.6

Analysis of Variance

RATS PER GROUP	VARIATION	DF	SUM OF SQUARES	MEAN SQUARE OR VARIANCE	F VARIANCE RATIO
4	between days	69	15487.48	224.46	2.595
	within days	84	7265.795	86.5	
8	between days	5	899.83	179.97	4.85
	within days	14	519.56	37.11	

were plotted together. When the dose-effect curves are parallel within the experimental error, the distance along the abscissa between the curve for urea and that for the substance gives the logarithm of the diuretic activity of the substance.

RESULTS. *Controls.* In the description of the method, special emphasis was placed on the necessity for using a sufficient number of rats in each group, and comparing the urinary excretion of the test groups with that of the control group of the same day, to ascertain that diuresis is obtained. Results presented in Table 1 give the comparison of the urinary excretion for groups on the *same* day

and on *different* days. The analysis of variance shows that the variation between days is significantly greater than between groups tested on the same day. As would be expected, the variance within days for groups of 8 rats is about one-half of that for groups of 4 rats.

Since the animals are fed a considerable amount of liquid at one time, it was of great importance to choose a liquid which is normally retained in the body to a large extent throughout the duration of the experiment, and which does not obscure the action of diuretics. Isotonic sodium chloride was found to fulfill these requirements. Substitution of Ringer's solution for saline does not significantly alter the value of urinary excretion. Substitution of tap water, however, more than doubles it (Table 2).

Urea. Since urea was chosen as the standard, it was more thoroughly studied than any other substance. The results obtained are summarized in Tables 3-5. Eight curves, each with three or four points, were obtained on different days using 8-rat groups. The data are given in Table 3. The variation in the effect

TABLE 2

Effect of substituting Ringer solution or tap water for saline solution on urinary excretion

Groups of eight rats each

EXPERIMENT DATE	URINARY EXCRETION						AVERAGES	
	Ringer solution			Control (saline)			Ringer solution	Control
1/ 7/42	54.1	37.5	42.5	50.5	52.1	47.2		
1/14/42	30.1	25.6		33.4	29.6			
1/15/42	33.1	30.0	50.2	33.1	35.4	28.3	36.8	37.9
	Tap water			Control (saline)			Tap water	Control
9/19/41	59.9	63.2		29.1	32.4			
12/ 9/41	65.4	65.5		23.0	29.7		63.5	28.6

expected for each day at the average dose of urea (25.3 millimoles per kg.) is greater than that in the slope of the dosage-effect curves, when both are compared with their respective errors. This difference in average susceptibility has been adjusted so as to bring the mean response-mean log-dose for each individual curve on the same line, Fig. 2, with a slope of b = .7005. This value agrees well with that obtained for 55 curves, each based on only 2 points (b = .7209) (Table 4, second row). The individual curves are relatively stable in slope with a smaller variance than that of individual readings about 7 of the 8 curves in Table 3. One curve, that on 11-30-42, was excessively variable. The mean slope in 63 experiments is 0.710 ± .026. An experiment was done with four different doses of urea on 4-rat groups, using the Latin-Square arrangement, and the results were analyzed by Yates' method (7) (Table 5). The variation due to the slope of the curve (b = .718) was greatest, that due to the difference in days was nearly 3 times as large as the error, but differences between rat groups and about the straight line were less than the experimental error.

Sodium, Potassium and Ammonium Salts. Of the five salts studied, potassium nitrate, potassium acetate and ammonium chloride are in common use as di-

TABLE 3

Dose-action curves from single experiments with 3 or 4 doses of urea; dose in millimoles of urea per kilogram of rat

DATE	PER CENT EXCRETION IN LOGARITHMS						SLOPE b	STANDARD DEVIATION ABOUT LINE s	LOG-EFFECT AT MEAN LOG-DOSE OF 1.4028
	Saline control	With log dose of urea							
		1.097	1.274	1.398	1.574	1.699			
11-5-41	1.617	1.769	1.867	1.898	2.064		.595	.0328	1.946
12-16-41	1.698	1.812		1.995	2.163	2.222	.703	.0236	2.021
12-17-41	1.643	1.855		2.037	2.152	2.288	.697	.0282	2.056
3-30-42	1.428	1.762*	1.815†	1.912			.848	.0232	1.893
11-27-42	1.732	1.655		1.785	1.949	2.179	.821	.0883	1.865
11-30-42	1.405	1.767		1.731	1.996	2.155	.643	.1328	1.885
12-1-42	1.555	1.718		1.830	2.036	2.191	.776	.0724	1.916
12-2-42	1.396	1.805		1.957	2.054	2.192	.615	.0401	1.975
Combined value							.7005	.0683	1.9446

* Log-dose = 1.223.
† Log-dose = 1.318.

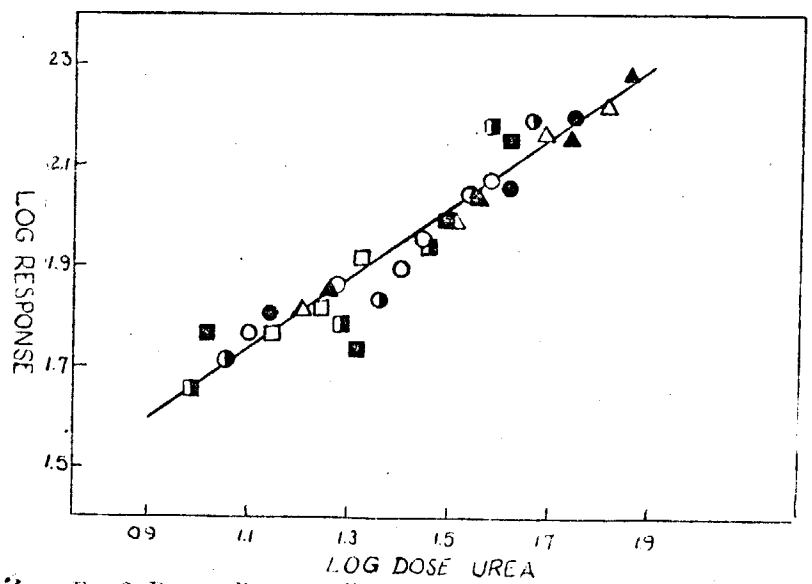


FIG. 2. DOSAGE RESPONSE CURVE FOR DATA ON UREA IN TABLE 3

The experiments on different days have been adjusted to an equal susceptibility for plotting by adding a constant term to the log-doses for each day. Each day's results are shown by a different symbol.

uretics, but not sodium nitrate or sodium acetate. The original data for potassium and sodium nitrate are given in Table 6, with the log-ratio of potencies (M)

TABLE 4

Analysis of variance between slopes of dose-action curves for urea in Table 3 and of 55 curves with 2 doses, not shown in detail; eight-rat groups on each dose

VARIATION DUE TO	DEGREES OF FREEDOM	MEAN SQUARES
Combined slope, all 63 curves of b_c = .71048	1	1.34948
Difference between 2 dose and multiple-dose slopes	1	.00028
Difference in slopes of individual curves	61	.00168
Scatter of observations about 7 of 8 separate curves in Table 3	13	.00267
Scatter of observations about curve for 11-30-42	2	.01763

TABLE 5

Experiment with 4-rat groups arranged in a Latin square as a test of sources of variation; highly variable controls omitted in analysis of variance by Yates' method

DATE	DIURETIC RESPONSE IN LOG OF % EXCRETION					TOTAL	SLOPE b
12/8	d 2.075	a 1.790	o (1.612)	b 1.902	c 1.989	7.756	.604
12/14	c 2.093	o (1.899)	b 2.037	d 2.135	a 1.821	8.086	.645
12/18	o (1.882)	c 1.853	d 2.150	a 1.597	b 1.982	7.582	1.019
12/23	a 1.954	b 2.088	c 2.022	o (1.814)	d 2.157	8.221	.364
12/28	b 1.738	d 2.094	a 1.655	c 1.930	o (1.213)	7.417	.960
Total	7.860	7.825	7.864	7.564	7.949	39.062	Mean .718

Dose of urea

	SYMBOL				
	o	a	b	c	d
	Log mM				
		1.097	1.273	1.398	1.574
Diuretic response in log of % excretion					
Total	8.360	8.817	9.747	9.887	10.611
Mean	1.6720	1.7634	1.9494	1.9774	2.1222

Analysis of variance

VARIATION DUE TO	DEGREES OF FREEDOM	MEAN SQUARE	F
Difference between groups of rats	4	.00534	.60
Difference between days of test	4	.02512	2.82
Slope of regression line	1	.31361	35.24
Curvature about line	2	.00616	.69
Error	8	.00890	1.00

computed for each assay. The data were first analyzed for the parallelism of their dose-response curves with the results shown in Table 6a. The small variance with few degrees of freedom about the individual curves is apparently not characteristic of the variation between curves. Although the 5 groups in Table 6 differ more in slope than the curves comprising the groups, the difference is not significant. Therefore, all observations have been combined to obtain a standard

TABLE 6

Dose-action curves of potassium nitrate and sodium nitrate and their activities relative to urea
M is log-ratio of potency relative to urea; M¹ that relative to KNO_3

GROUP	DRUG	LOG	LOG EXCRETION ON				GROUP	DRUG	LOG	LOG EXCRETION ON		
			10-1	10-2	11-10	11-11-41				12-1	12-2	12-3-41
		mM./kg.							*mM./kg.*			
1	KNO_3	.297			1.682		2	$NaNO_3$	.301	1.610		
		.393		1.731		1.770			.469		1.677	1.735
		.598			1.798				.582			1.820
		.694		1.989		1.945			.602	1.794		
		.773			1.961				.770	1.933	2.030	2.000
		.787	2.013						.946		2.196	2.163
		.870				2.061						
		1.092	2.266									
	Urea	1.319	1.863	1.828	1.863	1.845		Urea	1.319	1.904	1.904	
	Control		1.580	1.561	1.625	1.633		Control		1.486	1.584	1.539
	M		.722	.815	.702	.766		M		.606	.670	
			12-9	12-10	12-11-41					12-9	12-10	12-11-41
3	KNO_3	.393	1.618	1.493	1.808			$NaNO_3$	.389	1.595	1.553	1.598
		.870	2.066	2.018	2.106				.866	1.899	2.035	2.106
	Control		1.428	1.386	1.644			M¹		−.114	.033	−.126
			11-20	11-25	12-3-42					12-9	12-11	12-17-42
4	KNO_3	.490		1.705	1.742		5	$NaNO_3$	.469	1.733		
		.570	1.709						.645		1.724	1.761
		.787		2.066	1.938				.770	1.871		
		.870	1.936						.946		1.957	2.022
	Urea	1.097	1.636	1.791	1.810			Urea	1.097	1.843	1.734	1.815
		1.398	1.886	2.012	1.903				1.398	2.028	1.931	1.987
	Control		1.303	1.687	1.742			Control		1.695	1.632	1.694
	M		.604	.589	.588			M		.462	.462	.440

deviation of s = .0616, a combined slope of b = 0.8053 and λ = .0765. The log-ratio of potencies and its error is computed for each day with the combined slope. In this way the results are more consistent than when computed from the slopes for each group of tests (Table 7). The potencies of both KNO_3 and $NaNO_3$ relative to urea shift significantly between groups 1–2 and 4–5 in Table 6, but retain the same relation to each other throughout all tests as shown in the right-hand column of Table 7.

The dose-response curve of potassium acetate has the same slope as that of urea, so that its relative potency (avg. 3.35) is independent of the level of urinary excretion at which it is measured (Table 8). The dose-response curves of sodium acetate are, however, significantly steeper (b = 1.1194) than the corresponding curves of urea and potassium acetate (average b = 0.6085). In this case, there-

TABLE 6a

Analysis of variance for parallelism of dose-response curves in Table 6

VARIATION	DEGREES OF FREEDOM	VARIANCE	
Between slopes of dif. groups	4	.00599	
Between slopes within groups on dif. days	11	.00352	s^2 = .003794
Between slopes of dif. drugs on same day	9	.00316	
About 3 and 4 dose curves	6	.00056	

TABLE 7

Mean log-ratio of potencies based on Table 6

UREA AS STANDARD				POTASSIUM NITRATE AS STANDARD			
Group	Diuretic	M ± s_m	Relative potency	Groups	Diuretic	M ± s_m	Relative potency
1	KNO_3	.751 ± .046	5.63	1–2	$NaNO_3$	−.113 ± .078	.77
4	KNO_3	.594 ± .044	3.92	3	$NaNO_3$	−.069 ± .044	.85
2	$NaNO_3$	.638 ± .063	4.35	4–5	$NaNO_3$	−.139 ± .063	.73
5	$NaNO_3$	.455 ± .044	2.85				

TABLE 8

Dose-action curves of potassium acetate and its activity relative to urea

DRUG	LOG	LOG % EXCRETION ON						MEAN ACTIVITY
		10-3	11-7-41	12-21	12-22	12-29	12-30-42	
	mM./kg.							
K acetate	.582	1.796	1.723	1.774	1.927	1.609	1.772	3.35
	.805	1.951						
	.883		1.899	1.923	2.038	1.916	2.002	
	1.059		1.981					
Urea	1.097			1.668	1.888	1.784	1.843	1.00
	1.319	1.940	1.817					
	1.398			2.005	1.945	1.945	1.985	

fore, it is necessary to compute the relative potency from the data in Tables 9 and 10 at a specified level of log % excretion which is taken at 1.895. At higher levels of excretion, the relative potency of Na acetate would be greater relative to urea than at lower levels. In the later experiments it was significantly less than in the first tests in 1941.

The dose-response curve of ammonium chloride has the same slope (b = .8085, s = .0642) as that for urea (b = .6191, s = .1416), so that its relative potency, 2.7, is independent of the level of urinary excretion at which it is measured (Table 11).

Xanthine Diuretics. With theophylline and theobromine, an approximately linear relationship between log dose and log action is obtained only in the lower range of doses, "overdosage" producing a decrease in diuretic action. With caffeine, the measurable dose range is still more restricted. Calculation of the

TABLE 9

Dose-action curves of sodium acetate and its activity relative to urea and to potassium acetate

DRUG	LOG	LOG % EXCRETION ON										
		10-6	11-6	11-26	11-27	11-28	12-4	12-5	12-8	12-12-41	1-6	1-7-43
		1st group					2nd group				3d group	
	mM/kg.											
Na acetate	.662										1.557	
	.711						1.728	1.584	1.671	1.809		
	.741		1.611			1.766						1.530
	.866			1.915	1.930	1.940						
	.964	1.865			2.088	2.062					1.944	
	1.042		1.951	2.084	2.152							1.871
	1.063						2.017	2.032	2.000	2.161		
	1.167			2.253								
	1.220		2.118									
	1.265	2.152										
K acetate	.707						1.972	1.856	1.900	1.856		
	1.059						2.145	2.088	2.024	2.141		
Urea	1.097										1.856	1.671
	1.319	1.807	1.888	1.934	1.870	1.948						
	1.398										1.975	1.918

Mean activity of Na acetate
relative to urea.....(1st group)..... 2.762 ± .260
relative to K acetate.....(2nd group)..... .785 ± .045
relative to urea.....(3d group)..... 1.854 ± .106

diuretic activities is, therefore, possible only approximately. They are: theobromine, 7.2; caffeine, 32; and theophylline, 115. Theobromine in higher doses was fed in suspension, and in such doses it proved after 24 hours to be definitely toxic. With theophylline and caffeine, the doses could be increased to the desired extent, and the effect of overdosage could be studied in entirety. The highest dose used of theophylline produced a decrease in urinary excretion below that of the controls. Even with the lowest doses of theophylline and caffeine, there were definite manifestations of central excitation, but no such signs could be noticed with theobromine.

Bismuth Potassium Tartrate. The preparation used, with a Bi content of 62%, has a diuretic activity of approximately 219. High doses produce a decrease in urinary excretion, but even with diuretically active doses, albuminuria and glycosuria are observed.

Salyrgan. Salyrgan was tested alone and in combination with ammonium chloride. The results, given in figure 3, show salyrgan to be a potent diuretic

TABLE 10

Dose-action curves of sodium acetate and its activity relative to urea on 4-rat groups in the Latin Square arrangement

Since the analysis showed the differences between days relatively unimportant the results were handled as a randomized block.

GROUP	LOG % EXCRETION				
	Saline control	Acetate		Urea	
		.808	1.109	1.097	1.398
2	1.683	1.495	2.062	1.601	1.743
3	1.515	1.718	2.066	1.651	1.894
4	1.511	1.745	1.969	1.830	2.063
5	1.511	1.750	2.099	1.940	1.931
6	1.367	1.715	2.119	1.964	1.818
7	1.185	1.639	1.949	1.704	1.931
9	1.750	1.643	2.146	1.828	2.017
10	1.448	1.772	2.109	1.693	1.905
Total.....	11.970	13.477	16.519	14.211	15.302
Mean.....	1.4963	1.6846	2.0649	1.7764	1.9128

Relative potency = 1.982 ± .101.

Analysis of variance

	D.F.	MEAN SQUARE	F
Groups.....	7	.020980	2.76
Controls vs. treated.....	1	.845210	111.27
Acetate vs. urea.....	1	.007290	.96
Combined slope.....	1	.533803	70.27
Parallelism.....	1	.118950	15.66
Groups × control vs. treated.....	7	.028670	3.77
Error.....	21	.007596	1.00

within a very narrow range of doses. A slight overdose produces a definite decrease in urinary excretion. The active range is seemingly narrower without ammonium chloride and the optimum dose slightly higher. When ammonium chloride was used with salyrgan, in several instances the diuretic effect could be observed after five hours, and in nearly all instances, after ten hours. Without ammonium chloride, it took somewhat longer for the diuretic effect of salyrgan to become manifest. Within the range of diuretic doses, albuminuria was observed

in all instances and glycosuria in samples collected after ten hours. In the lower dose range, linear relationship was again obtained between the logarithm of the

TABLE 11

Dose-action curves of ammonium chloride and its activity relative to urea

DRUG	DOSE	LOG % EXCRETION ON								MEAN ACTIVITY
		2-23	3-2	3-3	3-10	3-24-42	4-17	5-	7-	
	mM/kg.									
NH_4Cl	.670	1.646								
	.711								1.647	
	.816			1.696	1.461	1.557	1.850	1.833		2.7
	.971	1.932	1.826	1.718						
	1.012								1.936	
	1.147		2.040	1.959	1.720	1.625	2.009	2.065		
Urea	1.097						1.731	1.735	1.724	
	1.319	1.838								1.0
	1.398						1.987	2.029	1.833	

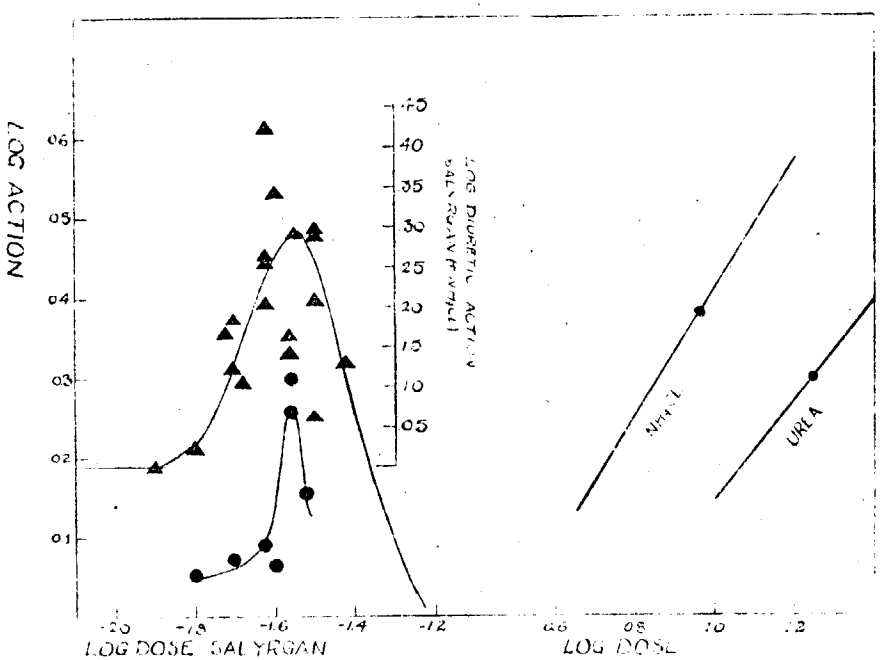


FIG. 3. DOSE ACTION CURVES OF AMMONIUM CHLORIDE, SALYRGAN, AND SALYRGAN WITH 4.67 mM/KG. AMMONIUM CHLORIDE, OVER A WIDE DOSE RANGE

●, salyrgan; ▲, salyrgan with NH_4Cl.

dose and the logarithm of action. The diuretic activity was found to be about 400.

Biuret. A number of substances which were considered likely to show some diuretic activity were studied. One of these is biuret which has a formula closely related to that of urea and is similar to it in some respects.[1] The original data obtained with low doses are given in Table 12. With higher doses necessitating a concentration greater than 5 per cent, biuret had to be fed in suspension. Due to its very steep solubility curve, it was practically impossible to make a homogenous suspension of biuret at the proper feeding temperature. Furthermore, when a concentration of 4 per cent or higher was used, biuret crystallized in the excreted urine at room temperature, clogging various parts of the metabolism cage. Therefore, the results obtained only with lower doses of biuret could be used for the calculation of the dose-action curve. This has the same slope (b = .5507, s = .0597) as that of urea (b = .5631, s = .0814). The mean potency of biuret relative to urea is 1.4.

DISCUSSION. The method described in this paper for assaying diuretics is significantly different from Burn's method for antidiuretics. It has proved to be applicable to the study of all types of diuretics in common use, and gives not only

TABLE 12

Dose-action curves of biuret and its activity relative to urea

DRUG	DOSE	LOG % EXCRETION ON							MEAN ACTIVITY
		12-22-41	1-27-42	1-28-42	1-19-43	1-22-43	1-26-43	1-28-43	
	mM/kg.								
Biuret	.779	1.611		1.733	1.641	1.666	1.705	1.696	
	.858		1.703	1.714					1.40
	.983		1.814	1.847					
	1.080	1.951	1.889	1.903	1.838	1.780	1.879	1.874	
Urea	1.097				1.706	1.795	1.786	1.837	
	1.319	1.913							1.00
	1.398				1.888	1.909	1.938	2.067	

an accurate estimate of the diuretic potency, but also some information concerning the therapeutic range and the toxic effects of the substance being studied. The limitations of this method, as of most bioassays on animals, arise from the fact that the results of assays on normal animals have to be compared with those on human patients. There is no question that the water-salt balance in the tissues of the normal rat is quite different from that in the tissues of the edematous patient. Nevertheless, it is remarkable that the sequence of activities of known diuretics is almost the same with the rat as it is with the human being: urea, ammonium chloride, potassium acetate, potassium nitrate, theobromine, theophylline, bismuth tartrate and salyrgan (Table 13). Caffeine, due to its very strong side effects, cannot be properly placed in this sequence. The relative potencies of the xanthine diuretics determined on rats, however, are in agreement with those determined by Myers on rabbits (8).

Though active in very small doses, the xanthine diuretics are not capable of

[1] Like urea, biuret will hemolyze erythrocytes in an isotonic solution and is distributed in equal concentration between outer fluid and cell.

producing as intense a diuresis in normal rats as the other substances studied. With high doses of urea or any of the neutral salts, it is possible to obtain within five hours a urinary excretion approximately twice the amount of the liquid administered. Likewise, with salyrgan, a urinary excretion of approximately the same magnitude may be obtained within the period of its activity. But with the xanthine diuretics, the amount of urine excreted within five hours is never significantly greater, and most frequently less than the amount fed. The shape and the scattering of the points of the dose-action curves, especially in the case of caffeine, suggest that there is a complex mechanism of xanthine diuresis and that a physiological limiting factor (e.g., the antidiuretic hormone) plays an important

TABLE 13

Comparison of diuretic activities from the dose-action curves and from human therapeutic doses

SUBSTANCE	AVERAGE SINGLE HUMAN DOSE			RAT DOSE FOR DIURETIC ACTION 1.5-2.5	DIURETIC ACTIVITY		RATIO RAT DOSE / HUMAN DOSE
					Human	Rat	
	gram	*gram/kg.*	*mM/kg.*	*mM/kg.*			
Urea	20–40	0.3–0.6	5–10	12–25	1	1	2.5
Biuret						1.4	
Sodium acetate						2.0	
Potassium acetate	15–30	0.23–0.46	2.4–4.8	4–8	2.1	3.4	1.7
Sodium nitrate						2.9	
Potassium nitrate	8–13	0.13–0.20	1.3–2.0	2.5–6.0	4	3.9	2.5
Ammonium chloride	10–20	0.15–0.30	2.8–5.6	4.6–8.9	2	2.7	1.6
		mg./kg.					
Theobromine	0.5–0.6	7.7–9.2	0.043–0.051	0.7–1.4	150	7.2	20
Caffeine	0.1	1.5	0.008	0.1–0.4	625	abt. 32	12–50
Theophylline	0.15–0.20	2.3–3.0	0.013–0.017	0.07–0.16	480	115	5–10
Salyrgan		2.4	0.004–0.008	0.027	1250	abt. 400	5
Bismuth Na (K) tartrate	0.03–0.09	0.5–1.5	0.0017–0.005	0.025–0.075	2000–3000	220	15

role; consequently these diuretics have variable effects on different species and even on different individuals.

Urea, biuret and the salts, with the possible exception of ammonium chloride, showed no toxic effects within the range of doses used. Consequently, the dose-action curves of these substances are linear within this range. The xanthine diuretics produced definite toxic effects in higher doses. The dose-action curves of these substances over a wide range of doses, showed some diuresis, and then a decline. The toxic effects of salyrgan are much more pronounced, and the range of diuretically active doses is much narrower for salyrgan than for the xanthine diuretics.

The following procedure is recommended for assaying new diuretics: Male rats weighing 140–240 gm. are fasted and deprived of water for 18 hours prior to the

experiment. Preliminary experiments are done using five groups of 8 rats each on one day. The control group are fed 25 cc./kgm 0.9% sodium chloride, containing no drug, and the remaining four groups are given geometrically increasing doses of the substance being tested. Thus the right dose range is determined in very few experiments. For the estimation of potency, five groups of 8 rats are used on one day. One group receives no diuretic, two receive two suitable doses of urea (e.g., 0.75 and 1.5 g./kgm.), and the remaining two receive two doses of the substance chosen so that their actions will fall within the same range as those of urea. The slope, position and the distance between the two regression lines are then calculated. Three or four such experiments will produce enough data for a sufficiently accurate estimate of the mean activity (potency) relative to urea of the substance.

SUMMARY

1. A method using white rats has been devised for the bioassay of diuretics. It has been tested experimentally and statistically and applied to the study of various substances. Urea with an activity of 1 was taken as the standard diuretic.

2. The dose-action curves (log dose in millimols per kilogram body weight, plotted against log diuretic action) of urea, various salts, and salyrgan were found to be straight lines of similar slopes within a suitable range of doses. From these the diuretic activities of the substances were calculated. The dose-action curves of the xanthine derivatives were found to be complex, seemingly limited by a physiological factor.

3. Biuret, a urea derivative, assayed in this way, showed a diuretic activity of 1.4.

4. The following diuretic activities were determined from the dose-action curves: salyrgan, 400; bismuth potassium tartrate, 219; theophylline, 115; caffeine, 32; theobromine, 7.2; potassium nitrate, 3.9; sodium nitrate, 2.9; potassium acetate, 3.4; sodium acetate, 2.0; ammonium chloride, 2.7; and urea, 1.0.

5. Relatively large doses of the salts and urea removed much more water than that which was administered, whereas such doses of the xanthine diuretics, Bi tartrate and of salyrgan not only failed to produce an increase in diuretic action, but in most instances produced a decrease.

REFERENCES

(1) Burn, J., Biological standardization, pp. 32 and 69, London, Oxford University Press, 1937.
(2) Marx, H., Arch. f. exper. Path. u. Pharmakol., **173**: 528, 1933.
(3) Herre, E., Arch. f. exper. Path. u. Pharmakol., **184**: 710, 1937.
(4) Jaretzky, R., and Neuwald, F., Arch. d. Pharmazie, **276**: 114, 1938.
(5) Vollmer, H., Arch. f. exper. Path. u. Pharmakol., **193**: 483, 1939.
(6) Ethridge, C. B., Meyers, D. W., and Fulton, M. N., Arch. Int. Med., **57**: 714, 1936.
(7) Yates, F.: J. Agricult. Science, **26**: part II, 301, 1936.
(8) Myers, H. B.: This Journal **23**: 465 and 477, 1924.

The authors are greatly indebted to Dr. C. I. Bliss for his advice and extensive help in the statistical analysis of the data.

THE EFFECTS OF ALKALOSIS UPON KETONE BODY PRODUCTION AND CARBOHYDRATE METABOLISM IN MAN

By S. R. LIPSKY,[1] B. J. ALPER,[2] M. E. RUBINI,[3] W. F. VAN ECK, and M. E. GORDON [4]

(*From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn., and the Veterans Administration Hospital, Newington, Conn.*)

(Submitted for publication February 11, 1954; accepted May 14, 1954)

J. Clin. Invest 33(9): 1269-1276, (1954)

Available evidence from animal investigation suggests that changes in pH can alter carbohydrate and ketone metabolism. Guest and Rawson (1) observed a distinct inverse relationship between blood sugar content and pH in rats. In the fasting animal, alkalosis was found to enhance ketosis and decrease liver glycogen (2). In human studies, Beumer and Soecknick (3) and Perges and Lipschütz (4) noted the early appearance of acetonuria in patients on subcaloric diets treated with sodium bicarbonate.

The present study was undertaken to define some of the metabolic changes attendant upon the acute administration of large amounts of alkali to normal and diabetic individuals in the postabsorptive state. Because of the known ketogenic activity (5) of acetic acid and the key position occupied by the 2-carbon fragment "acetate" in the intermediary metabolism of various foodstuffs (6), it was decided to compare the effect of alkalosis induced by sodium acetate to that of sodium bicarbonate.

EXPERIMENTAL PROCEDURES AND METHODS

An infusion of an isotonic solution consisting of 20.5 gm. of sodium acetate dissolved in 1600 cc. of distilled water was administered intravenously at a constant rate over a two hour period in all experiments. Eleven of the subjects, four medical students and seven patients hospitalized for minor ailments, were young adult males without evidence of disturbances of hepatic function or carbohydrate metabolism. Of the nine male diabetic subjects selected with ages ranging from 25 to 55 years, two patients, J. Z. and A. C., required no insulin, being controlled by diet alone. Both had undergone leg amputations for peripheral vascular disease six months prior to study. The diagnosis of diabetes mellitus was well substantiated before operation by the usual clinical and laboratory criteria. At the time of investigation both were found to be normoglycemic. The remaining seven subjects received no insulin for 2 to 5 days prior to observation. Several developed various degrees of diabetic ketosis during this period. One had mild associated acidosis. All subjects were maintained on diets containing adequate calories, protein, fat, and 250 grams of carbohydrate for at least one week prior to the study. Tests were routinely performed at 8 A.M. with the subjects in the postabsorptive state.

Analyses were performed before and at 120, 180, 240, and, in some instances, 280, 320, and 360 minutes after the onset of the infusion. Serum electrolytes were measured by methods previously described in publications from this laboratory (7). Citric acid in the serum was determined by the method of Natelson, Pincus, and Lugovoy (8). Blood was analyzed for total ketones, *i.e.*, the sum of acetone, acetoacetic acid, and β-hydroxybutyric acid by the method of Michaels, Margen, Liebert, and Kinsell (9), glucose by the iodometric titration method of Somogyi (10), and pyruvic acid by the technique of Friedemann and Haugen (11). When significant degrees of ketonemia were present, pyruvic acid filtrates were treated in a manner described by Lardy (12). Recoveries for glucose, ketones, citric and pyruvic acids ranged from 95 to 99 per cent in all instances.

Observations were made in two diabetic subjects, utilizing the hepatic vein catheterization technique of Bradley, Ingelfinger, Bradley, and Curry (13). Hepatic blood flow was determined by the bromsulphalein method as outlined by these authors.

Simultaneous samples of peripheral venous and arterial blood were obtained through indwelling Cournand needles placed in the left antecubital vein and brachial artery. Analyses of blood for glucose and ketones were performed in triplicate. The standard deviation of replicate samples for glucose at the 100 mgm. per cent level was ± 1 mgm.; for ketones at 1-2 mgm. per cent, ± 0.061; and at 20 mgm. per cent, ± 0.305 mgm. per cent.

Finally, eight paired control studies were performed in two consecutive days on four normal and four diabetic subjects. In one, sodium acetate, and in the other an equimolar solution of sodium bicarbonate was used.

RESULTS

Changes in acid-base balance

The administration of either sodium acetate or sodium bicarbonate solutions to normal and dia-

[1] U.S.P.H. Postdoctorate Research Fellow, National Heart Institute.

[2] U.S.P.H. Postdoctorate Research Fellow, National Institute of Arthritis and Metabolic Diseases.

[3] Captain, MC USA.

[4] Department of Medicine, Veterans Administration Hospital, West Haven, Conn.

TABLE I

Serum CO_2 and chloride response to the intravenous administration of equimolar quantities of Na acetate and $NaHCO_3$ in normal and diabetic subjects *

Time	Na Acetate						$NaHCO_3$					
	Normals			Diabetics			Normals			Diabetics		
	No.	CO_2	Cl	No.	CO_2	Cl	No.	CO_2	Cl	No.	CO_2	Cl
min.		*mEq./L.*			*mEq./L.*			*mEq./L.*			*mEq./L.*	
0	11	25.2 ±0.177	95.1 ±0.812	8	24.7 ±0.453	91.2 ±0.988	6	25.6 ±0.486	95.3 ±1.36	6	24.2 ±0.583	91.1 ±1.88
120		30.5 ±0.291	88.9 ±0.915		29.5 ±0.517	88.7 ±0.877		31.3 ±0.482	89.3 ±1.53		29.1 ±0.538	87.1 ±1.63
240		28.8 ±0.389	90.5 ±1.90		27.7 ±0.692	90.3 ±1.06		29.8 ±0.408	90.5 ±1.65		28.3 ±0.772	89.3 ±0.808

* The mean and standard error.

betic subjects produced comparable degrees of metabolic alkalosis. Table I summarizes the changes observed in all studies including the paired experiments. Alkalosis was maximal at the termination of the infusion (120 minutes) and still persisted at 240 minutes, but to a lesser extent. Simultaneously, the chloride concentration of the serum decreased to a roughly equivalent degree in all cases.

Ketones

Two diabetic patients, L. R. and D. B., were subjected to hepatic vein catheterization prior to the administration of sodium acetate (Figures 1, 2). The initial ketone concentration in the blood of L. R. was slightly elevated. D. B. was in moderate diabetic ketosis at the onset of the procedure. The splanchnic ketone output which was determined by multiplying the hepatic vein-arterial

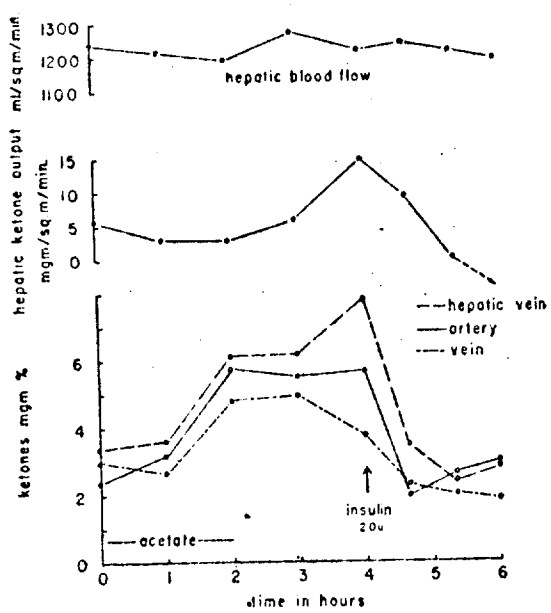


FIG. 1. PATIENT L. R. WITH MILD DIABETES. THE EFFECT OF ACETATE AND INSULIN ON HEPATIC BLOOD FLOW AND HEPATIC KETONE PRODUCTION

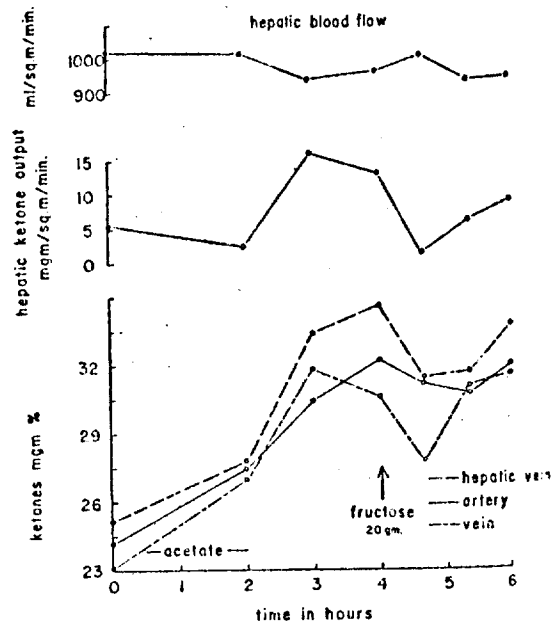


FIG. 2. PATIENT D. B. WITH SEVERE DIABETES. THE EFFECT OF ACETATE AND FRUCTOSE ON HEPATIC BLOOD FLOW AND HEPATIC KETONE PRODUCTION

(HV-A) ketone difference by the estimated hepatic blood flow was quantitatively similar in both instances. Ketone production rose from 5 mgm. per M^2 per minute to 15 mgm. per M^2 per minute at 180 (D. B.) and 240 minutes (L. R.) after the onset of the infusion. The greatest HV-A differences were apparent at these points. The largest peripheral arterial-venous (A-V) differences were noted at 240 minutes. The hepatic blood flow remained relatively constant throughout.

Eighty minutes after the intravenous administration of 20 units of regular insulin to subject L. R., splanchnic ketone production as evidenced by HV-A differences fell, approaching zero. The concentration of ketones in the hepatic vein, peripheral artery, and vein fell to initial levels. These effects persisted until the termination of the experiment.

Likewise, 40 minutes after the rapid intravenous injection of 20 grams of fructose to patient D. B., a sharp depression in splanchnic ketone output was noted. The peripheral A-V difference increased significantly at this point, indicating increased peripheral ketone utilization. However, these effects were only fleeting, for 80 and 120 minutes later ketone production was again approaching precarbohydrate injection levels. Similar changes are reflected in the ketones of the hepatic vein, peripheral artery, and vein.

The intravenous infusion of 100 grams of fructose over a 60 minute period to a patient in diabetic ketosis, not treated with acetate, resulted in a similar decline followed by a secondary rise above preinjection ketone concentrations (14).

The response of blood ketones to intravenously administered sodium acetate and sodium bicar-

TABLE II

The blood ketone response to intravenous administration of sodium acetate and sodium bicarbonate in normal and diabetic subjects

Normals	Time in minutes 0	120	180	240
	mg. %			
Na Acetate				
F. G.	1.6	1.8	1.6	2.9
S. D.	2.0	3.0	4.7	4.3
G. M.	2.1	2.7	3.3	4.0
H. D.	2.0	2.0	2.9	3.6
T. H.*	2.0	2.0	1.6	2.9
E. F.*	1.5	2.0	1.9	2.8
F. J.*	1.5	1.6	2.7	2.7
K. H.*	1.4	1.2	1.4	2.5
Mean	1.8	2.0	2.5	3.2
Standard Error	±0.102	±0.58	±1.13	±0.235
$NaHCO_3$				
R. S.	1.6	1.6	1.7	2.1
L. L.	1.7	2.4	1.9	2.0
T. H.*	1.8	1.7	1.7	1.4
E. F.*	2.4	1.4	1.2	0.9
F. J.*	1.4	1.1	0.9	0.8
K. H.*	1.6	1.5	1.4	1.1
Mean	1.8	1.6	1.6	1.4
Standard Error	±0.143	±0.179	±0.163	±0.229

Diabetics	Time in minutes 0	120	180	240
	mg. %			
Na Acetate Mild Diabetics				
L. R.	3.0	4.8	5.0	3.8
A. C.	1.3	1.7	2.8	2.9
F. H.*	2.0	2.8	3.8	3.7
L. D.*	3.2	2.8	3.4	4.4
J. S.*	0.7	1.7	2.5	1.9
Mean	2.0	2.8	3.5	3.5
Standard Error	±0.480	±0.565	±0.04	±0.43
Na Acetate Severe Diabetics				
H. B.	16.0	25.3	26.6	29.2
D. B.*	23.0	26.9	31.8	30.6
D. B.	10.7	14.1	17.2	16.4
L. B.*	27.4	38.8	40.5	46.7
Mean	19.3	26.2	29.0	30.7
Standard Error	±3.7	±5.0	±5.0	±6.2
$NaHCO_3$ Mild Diabetics				
F. H.*	4.1	3.5	5.5	3.1
L. D.*	3.2	2.3	3.1	2.7
J. S.	1.9	2.3	2.4	2.5
H. B.	3.2	2.3	6.1	7.0
Mean	3.1	2.6	4.3	3.8
Standard Error	±0.15	±0.30	±0.72	±1.07
$NaHCO_3$ Severe Diabetics				
D. P.*	15.3	30.3	42.3	42.2
L. B.*	23.2	26.7	29.1	33.1

* Paired studies.

bonate in normal and diabetic subjects are presented in Table II. In both single and paired observations in the normal group, following acetate infusions, blood ketones increased slightly, but significantly ($p < 0.01$) and consistently. In the control series receiving bicarbonate there were no similar changes. Indeed, in the majority of subjects, ketone concentrations diminished slightly and progressively. A comparison of the mild diabetics at 0 and 240 minutes shows a significant increase in ketones following acetate infusions, but a borderline or insignificant increase following bicarbonate. In severe diabetics, ketones increased appreciably following infusions of either acetate or bicarbonate. The absolute rise was greatest in those individuals who were initially in diabetic ketosis.

Glucose

The effects of acetate on the splanchnic glucose metabolism of the two diabetic subjects, L. R. and D. B., are shown in Figures 3 and 4. In both individuals, splanchnic glucose output decreased

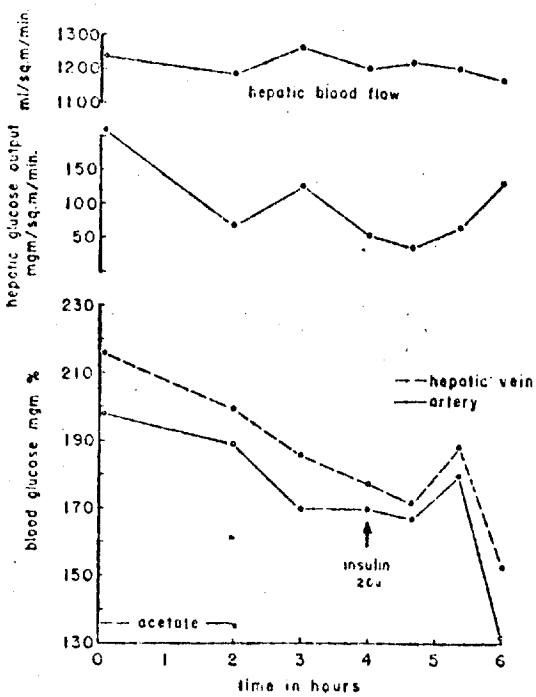


FIG. 3. PATIENT L. R. WITH MILD DIABETES. THE EFFECT OF ACETATE AND INSULIN ON HEPATIC BLOOD FLOW AND HEPATIC GLUCOSE PRODUCTION

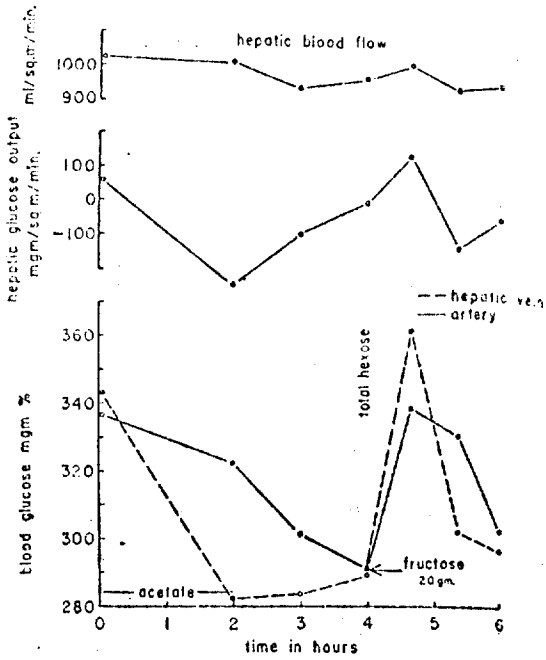


FIG. 4. PATIENT D. B. WITH SEVERE DIABETES. THE EFFECT OF ACETATE AND FRUCTOSE ON HEPATIC BLOOD FLOW AND HEPATIC GLUCOSE PRODUCTION

strikingly. In patient L. R. this was most marked at 120 and 240 minutes. Two hours after the intravenous administration of 20 units of insulin, the blood sugar of this patient had fallen approximately 40 mgm. per cent with an associated increase in splanchnic glucose production. The action of insulin on peripheral blood sugar concentrations and splanchnic glucose output was similar to that observed by Bearn, Billing, and Sherlock (15). In subject D. B. glucose production fell precipitously. The splanchnic bed began to remove glucose from the circulation, producing negative values for HV-A. This was most pronounced at the completion of the acetate infusion and persisted to a lesser degree for an additional 120 minutes. However, with the passage of time, there was a tendency for the glucose concentration in the hepatic vein to rise again above that in the artery. At 240 minutes the rapid intravenous injection of 20 grams of fructose induced an outpouring of hexose into the blood stream by the liver. This phenomenon was dissipated within a 40 minute period, and only after peripheral concentrations began to descend again was an upward trend in output seen.

TABLE III

The response of blood glucose to intravenous administration of sodium acetate and sodium bicarbonate in normal and diabetic subjects

Normals	Time in minutes 0	120	180	240	Diabetics	Time in minutes 0	120	180	240
	mg. %					mg. %			
Na Acetate					Na Acetate				
W. J.	84	82	84	82	A. P.	201	186	184	174
F. G.	80	82	83	77	L. R.	215	193	178	168
S. D.	67	67	72	70	E. B.	184	164	163	167
G. M.	63	69	73	74	H. B.*	237	203	200	203
H. D.	63	62	71	77	D. B.*	437	382	393	389
T. H.*	74	73	76	82	D. B.	339	323	297	292
E. F.*	78	76	81	81	F. H.*	212	190	176	162
K. H.*	75	70	78	81	L. D.	172	149	143	141
F. J.*	76	73	80	73	J. Z.†	86	87	88	86
					A. C.†	92	88	91	94
Mean	73	73	77	78	Mean	217	196	191	187
Standard Error	±2.17	±2.54	±1.60	±1.47	Standard Error	±33.3	±29.4	±28.9	±28.7
$NaHCO_3$					$NaHCO_3$				
R. S.	84	86	87	84	H. B.*	241	177	199	186
L. L.	102	106	110	110	D. B.*	436	415	379	367
T. H.*	75	74	71	70	F. H.*	207	157	162	161
E. F.*	82	81	80	84	L. D.*	218	182	180	175
K. H.*	79	77	80	72	J. Z.†	104	101	107	100
F. J.*	72	78	71	69	A. C.†	83	77	79	92
Mean	82	84	83	82	Mean	215	185	183	181
Standard Error	±4.32	±4.73	±5.69	±6.33	Standard Error	±51.7	±49.2	±43.0	±40.5

* Paired studies.

† Normoglycemic at time of study, had substantiated diagnosis of diabetes, and required insulin prior to leg amputations for peripheral vascular disease.

Table III shows the changes observed in the glucose of the blood following the infusion of acetate and bicarbonate to normal and diabetic individuals. The blood sugar of the normal subjects did not change appreciably in single and paired studies after the administration of either solution. In the diabetic group, however, at the termination of the experiment, mean decreases of 30 and 34 mgm. per cent below initial concentrations were noticed after the injection of acetate and bicarbonate, respectively. There was no distinct correlation between the height of the initial blood sugar and the degree of depression at the termination of the experiment.

Citric acid

Citric acid concentrations in the blood increased significantly in both normal ($p < 0.02$) and diabetic subjects ($p < 0.02$) at 240 minutes following the infusion of acetate (Table IV). Small increments noted after the bicarbonate solution were not statistically significant. In this group, at 180 minutes, two subjects showed peak increases of similar magnitude to those seen in the acetate treated series. However, these increments were followed by slight declines at the 240 minute pe-

TABLE IV

Blood citric acid response to the intravenous administration of Na acetate and $NaHCO_3$ in normal and diabetic subjects *

Time	Na Acetate				$NaHCO_3$			
	Normals		Diabetics		Normals		Diabetics	
	No.	Citric acid	No.	Citric acid	No.	Citric acid	No.	Citric acid
0	5	2.30 ±0.187	7	2.28 ±0.098	6	2.09 ±0.204	6	2.33 ±0.094
120		2.13 ±0.295		2.12 ±0.105		1.91 ±0.231		2.09 ±0.229
180		2.69 ±0.241		2.59 ±0.375		2.35 ±0.273		2.39 ±0.114
240		2.50 ±0.053		2.78 ±0.111		2.03 ±0.219		2.58 ±0.081

* Mean and standard error of mean.

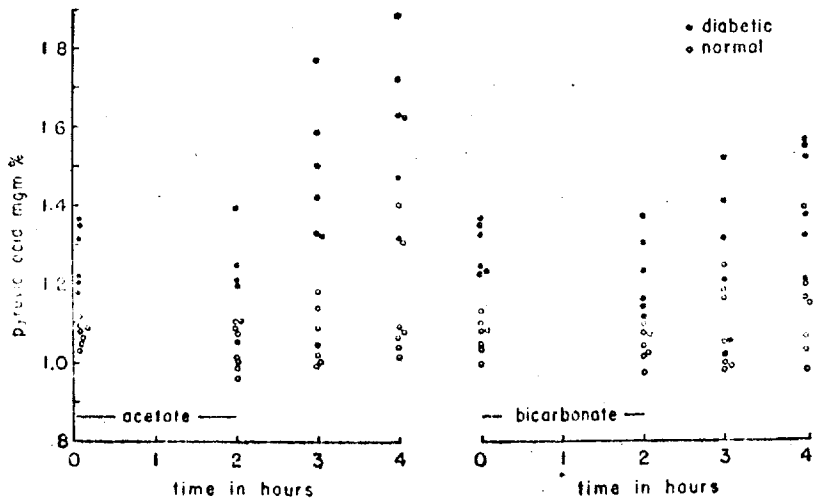


FIG. 5. THE EFFECTS OF ACETATE AND BICARBONATE ADMINISTRATION ON BLOOD PYRUVIC ACID CONCENTRATION IN NORMAL AND DIABETIC SUBJECTS

riod. The initial values of both groups fell within the normal range for blood citric acid concentration.

Pyruvic acid

The administration of acetate or bicarbonate to normal subjects (Figure 5) usually resulted in a slight decline of blood pyruvate at the termination of the experiment. In two instances, using either solution, pyruvate concentrations rose at this point. A more definitive pattern was noted in the diabetic group of patients (Figure 5). Generally, slight depressions in pyruvic acid at the end of the infusion period were followed by subsequent increases above initial concentrations. In the acetate treated group this elevation (12 to 38 per cent) appeared earlier, was higher and more consistent than that noted in subjects receiving bicarbonate.

The patients with diabetes mellitus exhibited slightly higher pre-injection concentrations than the normal group.

DISCUSSION

Acetate as such is not found in the blood stream under normal conditions. The rapidity with which this compound is metabolized in the mammalian body has been demonstrated by Harper, Neal, and Hlavacek (16). Following the rapid intravenous injection of C^{14} labeled acetate (4.8 mM per Kilo, in 1 to 2 mins.) to dogs, these investigators noted the quick appearance and maximal concentration of C^{14} in the CO_2 of the expired air within 30 minutes after administration. During the same period, the formation of new $C^{14}O_2$ ceased as the tagged acetate rapidly disappeared from the blood. This was taken as presumptive evidence for utilization of this compound. If the data obtained from these experiments are applied to our studies, it can be assumed that at the rate of administration of 15 grams of exogenous acetic acid (20.5 grams CH_3COONa or 3.5 mM per Kilo) over 120 mins., the metabolism of 2C fragments was almost instantaneous. However, the amount of acetate that could be given was limited by the associated sodium load. Under these circumstances the alkalosis that was produced was comparable to that induced by equimolar amounts of $NaHCO_3$. With the rapid utilization of the acetate radical, the associated sodium ions became available for combination with CO_2 and H_2O. The effect in each instance, therefore, was an increase in the concentration of sodium bicarbonate in the serum. Since it is virtually impossible to differentiate the metabolic effects of acetate "loading" from those of concomitant alkalosis, it seems reasonable to conclude that the results obtained in this study probably were related to alterations in the pH of body fluids *per se*.

Alkalinization ($NaHCO_3$ or Na acetate) in the diabetic subject was associated with a fall in blood sugar and an accentuation of pre-existing ketosis (Tables II, III). In the two diabetic patients (Figures 3–4) subjected to hepatic vein catheterizations, splanchnic glucose production decreased and hepatic ketone output increased following the administration of sodium acetate. From the results of previous animal investigations on the effects of alkalosis on carbohydrate, protein, and fat metabolism (2), it appears that these changes are, for the most part, directly related to a decrease in the transformation of protein to glucose. Since ketosis varies inversely with the availability of carbohydrate, the amount of fat converted to ketone bodies in the diabetic becomes largely dependent upon the extent and rate at which these catabolic processes take place. In the acetate experiments, it is conceivable that the addition of 2C fragments could alter total body metabolism. Fifteen grams of acetate given over two hours would represent an appreciable fraction of the caloric requirement during this period of time. For example, if this rate of administration were continued over 24 hours, 180 grams of acetate could provide over one-half the total fat and carbohydrate intake per day in equivalent weight of carbon chains. Two carbon fragments are rapidly converted into ketone bodies in the uncontrolled diabetic. Although ketone bodies can readily be utilized to meet, in part, the energy requirements of the body, it is improbable that this effect alone could account for the diminution in the production of glucose from various sources, since similar results were noted following bicarbonate administration.

The lack of appreciable changes in glucose concentration noted during alkalosis in the normal subjects, and in the two diabetic patients, J. Z. and H. C., who became normoglycemic following leg amputations for obliterative peripheral vascular disease, remains unexplained. It may be hypothesized that the observed fall occurs only in the presence of either overproduction of glucose from noncarbohydrate sources or inadequate stores of liver glycogen. However, the latter seems unlikely, for in a normal subject fasted for 48 hours and whose liver glycogen was presumably depleted, the blood sugar remained relatively unchanged while ketosis was enhanced with alkalosis (14).

In eight paired studies ketone concentrations in the blood of normal subjects failed to increase following the administration of $NaHCO_3$ (Table II). The individuals in these experiments were in the postabsorptive state. No prior stimulus for ketosis existed. However, it has been noted that in the presence of depleted carbohydrate stores, alkalosis induced by either $NaHCO_3$ (3, 4) or hyperventilation (3, 17) will accentuate ketosis. In the latter situation, respiratory alkalosis was associated with an actual decrease in serum bicarbonate. In the acetate treated group of normals the slight but significant increase in ketone bodies probably represents the result of condensation of small excesses of preformed acetate.

It has long been known that shifts in pH toward alkalinity are associated with substantial increments of citric acid in blood and urine (18). The efficiency of the reaction acetoacetate + oxaloacetate → citric acid may therefore be considerably increased during alkalosis. Thus in the subjects receiving acetate, the mechanisms for the increased production of citric acid may occur singly or in combination. First, it may be an effect of alkalosis alone; second, it may be due to the availability of excesses of acetate or acetoacetate in the presence of intracellular oxaloacetate. The slight fall in citric acid noted in several studies coincident with maximal alkalosis at the termination of the infusion at 120 minutes probably represents a distribution phenomenon.

Very little is known concerning the pathways of pyruvate metabolism in man. The somewhat variable changes that were noted in blood pyruvate can not be explained at this time in the light of present knowledge.

SUMMARY

1. The administration of large amounts of acetate as the sodium salt to normal and diabetic subjects results in the production of an alkalosis comparable to that induced by equimolar solutions of $NaHCO_3$.

2. In normal subjects there were no changes in blood glucose concentrations following the infusion of acetate or bicarbonate.

3. The administration of sodium acetate to diabetic subjects caused decreases in splanchnic glucose output and peripheral blood glucose concentrations. These effects were associated with simul-

taneous increases in splanchnic ketone production and peripheral blood ketones. Diabetics treated with bicarbonate usually exhibited similar changes in peripheral glucose and ketone levels.

4. The effect of alkalosis *per se* on carbohydrate metabolism is discussed.

REFERENCES

1. Guest, M. M., and Rawson, R. A., Carbohydrate storage and mobilization with changes in the blood pH. J. Biol. Chem., 1941, **139**, 535.
2. MacKay, E. M., Wick, A. N., Carne, H. O., and Barnum, C. P., The influence of alkalosis and acidosis upon fasting ketosis. J. Biol. Chem., 1941, **138**, 63.
3. Beumer, H., and Soecknick, A., Über organische Acidose bei anorganischer Acidose und Alkalose. Ztschr. f. Kinderh., 1924, **37**, 236.
4. Porges, O., and Lipschütz, H., Über Azetonurie und Alkalose. Arch. f. exper. Path. u. Pharmakol., 1923, **97**, 379.
5. MacKay, E. M., Barnes, R. H., Carne, H. O., and Wick, A. N., Ketogenic activity of acetic acid. J. Biol. Chem., 1940, **135**, 157.
6. Engel, F. L., The endocrine control of metabolism. Bull. New York Acad. Med., 1953, **29**, 175.
7. Peters, J. P., and Van Slyke, D., Quantitative Clinical Chemistry, 2nd ed., Volume II. The Williams and Wilkins Co., Baltimore, 1946.
8. Natelson, S., Pincus, J. B., and Lugovoy, J. K., Microestimation of citric acid; a new colorimetric reaction for pentabromoacetone. J. Biol. Chem., 1948, **175**, 745.
9. Michaels, G. D., Margen, S., Liebert, G., and Kinsell, L. W., Studies in fat metabolism. I. The colorimetric determination of ketone bodies in biological fluids. J. Clin. Invest., 1951, **30**, 1483.
10. Somogyi, M., Determination of blood sugar. J. Biol. Chem., 1945, **160**, 69.
11. Friedemann, T. E., and Haugen, G. E., Pyruvic acid II. The determination of keto acids in blood and urine. J. Biol. Chem., 1943, **147**, 415.
12. Lardy, H. A., Method for pyruvic acid *in* Manometric Techniques and Related Methods for the Study of Tissue Metabolism, Minneapolis, Burgess Publishing Co., 1945, Chapter X, p. 105.
13. Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J., The estimation of hepatic blood flow in man. J. Clin. Invest., 1945, **24**, 890.
14. Personal observations.
15. Bearn, A. G., Billing, B. H., and Sherlock, S., The response of the liver to insulin in normal subjects and in diabetes mellitus: hepatic vein catheterization studies. Clin. Sc., 1952, **11**, 151.
16. Harper, P. V., Jr., Neal, W. B., Jr., and Hlavacek, G. R., Acetate utilization in the dog. Metabolism, 1953, **2**, 62.
17. Davies, H. W., Haldane, J. B. S., and Kennaway, E. L., Experiments on the regulation of the blood's alkalinity. J. Physiol., 1920, **54**, 32.
18. Sherman, C. C., Mendel, L. B., and Smith, A. H., The citric acid formed in animal metabolism. J. Biol. Chem., 1936, **113**, 247.

Chin. J. Sci. Agric. 1: 173-178, 1944

THE PEPTIZATION OF RICE PROTEINS BY ORGANIC ACIDS AND ORGANIC SALTS

Teng-Yi Lo and Chih-Hua Wu[1]

Gortner and his associates (3,4,5,7,8) have found wide differences in the peptizing capacity of inorganic salt solutions for the proteins in various seeds and grains. It was pointed out that the colloid chemical viewpoint affords the most satisfactory explanation for many of the properties of protein systems.

Mangels and Martin (6) reported the peptizability of wheat flour proteins in organic acids, and found that three factors affected the dispersing or peptizing power of the organic acids, namely, H-ion concentration, number of carbon atoms, and presence or absence of hydroxy groups.

From the review of literatures it was found that no systematic study along this line has ever been made of the rice proteins. The present investigation was undertaken to determine if the rice proteins would show similar variations in peptizability.

MATERIAL AND METHODS

Four samples used in this study were so chosen as to represent four distinct varieties of rice, differing widely in physical and chemical characteristics, namely, the early non-glutinous rice (早籼稻), late non-glutinous rice (晚籼稻), semi-non-glutinous rice (糯稻), and yellow-silky glutinous rice (黃絲糯). In our experiments it was thought best, whereever possible, to grind the samples sufficiently fine that they would all pass a 80-mesh sieve.

Seven comparatively common organic acids, namely, acetic, propionic, lactic oxalic, succinic, tartaric and citric acid, and four sodium salts of organic acids, namely, acetate, oxalate, tartrate and citrate were used for this study. In this group of acids are represented mono-basic, di-basic, and tri-basic acids, and, also, three hydroxy acids. The majority of the acids and salts were used in four concentrations—0.1, 0.5, 1.0, and 2.0N.

The method of peptization of rice proteins used was briefly as follows: One gram of rice meal was weighed in a 250 c.c. Erlenmeyer flask and 50cc. of the acid or salt added from a pipette. The flask was stoppered and thoroughly shaken at

1. Biochemical and Nutritional Laboratory, Department of Agricultural Chemistry, The National Chekiang University, Meitan, Kweichow.

-minute intervals for 3 hours, at room temperature. At the end of 3 hours, the e-meal suspension was filtered thru a dry folded filter, and with a pipette roduced 20 cc. of the filtrate into a Kjeldahl flask. Nitrogen was then determined the Cavett's micro-Kjeldahl method (2), and the copper-selenium phosphoric-lfuric acid mixture by the modified method of Campbell and Hanna (1) was ed as the acid digesting agent. Nitrogen and moisture were determined in the ce-meal samples and the acid or salt extraction data calculated on the moisture-ee basis as per cent of total potein peptized by the acid or salt. Duplicate tractions were made in all instances.

RESULTS AND DISCUSSION

The experimental data, expressed in percentage of total protein peptized from ce meals by different organic acids and salts in various concentrations are shown tables 1 and 2.

The influence of H-ion concentration, number of carbon atoms, and hydroxy groups peptization. Table 3 shows the H-ion concentration (as pH, determined olorimetrically) of the organic salt solutions and of the salt solution extracts of ne rice-meals.

The data of table 1 do not support the view of Mangels and Martin (6) that the eptizating capacity of the organic acids varied inversely as the H-ion concentra-ion, and it is perfectly obvious from an inspection of tables 2 and 3 that no orrelation between pH of the organic salt solution extract of the rice-meal and he percent of protein peptized is found. Accordingly, the H-ion concentration is ot playing a major role in the peptizing action of the organic acids and salts.

Furthermore, the results obtained do not indicate that the introduction of CH_2 roup into the molecule of organic acids decreased their peptizing power and ne introduction of hydroxy groups increased the peptizing capacity of organic cids. This fact is not in agreement with that reported by Mangels and Martin (6) lso.

The influence of rice variety on peptization. Table 4 records the varietal ariation in peptization by organic acids at 0.5 N concentration for the four arieties of rice. The yellow-silky glutinous rice showed the highest percentage of rotein peptized by organic acids and the early non-glutinous rice the lowest. The rder for all the acids used is the yellow-silky glutinous rice, late non-glutinous ice, semi-non-glutinous rice, and early non-glutinous rice; for mono-basic acids is ne yellow-silky glutinous rice, late non-glutinous rice and early non-glutinous rice; or di-basic acids is the yellow-silky glutinous rice, semi-non-glutinous rice, late on-glutinous rice, and early non-glutinous rice; and for hydroxy acids is the ellow-silky glutinous rice, late non-glutinous rice, semi-non-glutinous rice and early non-glutinous rice.

TABLE 1. *Percentage of total protein peptized from rice meals by different organic acids in various concentrations (on dry basis)*

Rice variety	Moisture (%)	Total nitrogen based on		Total protein based on		Percent of total protein peptized by indicated acids								
						Acetic acid				Propionic acid		Oxalic acid		
		air dry weight (%)	dry weight (%)	air dry weight (%)	dry weight (%)	0.1N	0.5N	1.0N	2.0N	0.5N	1.0N	0.1N	0.5N	1.0N
[illegible]y non-glutinous	10.71	1.283	1.540	8.001	9.025	1.098	5.050	8.424	3.085	0.101	15.800	5.725	18.420	8.025
[illegible] non-glutinous	17.02	1.039	1.322	6.806	8.243	5.149	7.200	7.103	9.024	18.110	15.370	9.158	11.293	11.810
[illegible]rage of the above 2 varieties	—	—	—	—	—	3.459	6.200	8.102	6.210	17.242	14.020	7.442	15.[illegible]	9.010
[illegible]-non-glutinous	16.81	1.102	1.324	6.558	8.275	3.093	4.570	9.070	4.078	10.700	8.418	7.810	12.195	15.2[illegible]
[illegible]-silky glutinous	16.43	1.210	1.458	8.483	9.958	5.194	14.010	22.010	24.725	17.810	28.810	19.210	25.075	19.0[illegible]
[illegible]rage of all 4 varieties	—	—	—	—	—	4.038	7.577	11.017	10.456	18.014	17.810	9.007	17.147	11.928

TABLE 1. *Percentage of total protein peptized from rice meals by different organic acids in various concentrations (on dry basis)—continued*

[illegible]	Percent of total protein peptized by indicated acids														
	Lactic acid				Succinic acid			Tartaric acid				Citric acid			
	0.1N	0.5N	1.0N	2.0N	0.1N	0.5N	0.8[illegible]	0.1N	0.5N	1.0N	2.0N	0.1N	0.5N	1.0N	2.0N
[illegible]	2.102	10.915	11.1[illegible]	21.010	1.2[illegible]	5.227	7.[illegible]	[illegible].625	10.915	5.008	20.010	4.1[illegible]	10.5[illegible]	[illegible]	15.170
[illegible]	5.775	12.140	18.120	27.100	3.514	8.429	12.765	[illegible]	11.255	14.510	27.055	9.5[illegible]	13.[illegible]	11.020	19.040
[illegible]	3.910	12.028	14.885	22.095	2.[illegible]	6.[illegible]	10.010	7.[illegible]	11.071	20.059	[illegible]	[illegible]	11.[illegible]	12.040	17.108
[illegible]	4.751	9.515	12.210	14.010	1.[illegible]	8.482	11.[illegible]	4.804	11.470	8.170	22.410	6.[illegible]	7.[illegible]	7.[illegible]	10.082
[illegible]	14.970	22.005	18.010	41.210	3.1[illegible]	10.205	17.710	9.5[illegible]	14.580	20.115	19.[illegible]	7.157	10.010	17.075	12.110
[illegible]	6.500	14.354	17.514	21.070	[illegible]	[illegible]	12.208	[illegible]	15.210	18.802	[illegible]	[illegible]	11.419	[illegible]	10.204

a. Approximately

TABLE 3: *H-ion concentration of organic salt solutions and salt solution extracts of rice meals*

Rice Variety	Na-acetate						Na2-oxalate				Na3 citrate						Na2-tartrate					
	0.1N		0.5N		1.0N		0.1N		0.5N		0.1N		0.5N		1.0N		0.1N		0.5N		1.0N	
	Soln.	Extr.	Soln.	Extr.	Soln.	Extr.	Soln.	Extr.	Soln.	Extr.	Soln.	Extr.	Soln.	Extr.	Soln.	Extr.	Soln.	Extr.	Soln.	Extr.	Soln.	Extr.
Non-glutinous	8.3	7.3	8.4	7.9	8.6	8.3	7.3	7.0	7.5	7.3	8.4	7.6	8.2	7.8	8.0	7.8	6.9	6.9	6.9	6.9	7.0	6.9
Semi-non-glutinous	8.3	7.1	8.4	7.9	8.6	8.2	7.3	7.0	7.5	7.4	8.4	7.8	8.2	8.0	8.0	7.8	6.9	6.9	6.9	6.9	7.0	6.9
Yellow-silky glutinous	8.3	8.1	8.4	8.1	8.6	8.2	7.3	7.2	7.5	7.5	8.4	[illegible]	8.2	8.0	8.0	7.8	6.9	7.0	6.9	7.0	7.0	7.0

Soln.=Salt solution. Extr.=Salt solution extract.

TABLE 2: *Percentage of total protein peptized from rice meals by different organic salts in various concentrations (on dry basis)*

Rice Variety	Percent of total protein peptized by indicatde salts										
	Na-acetate			Na2-oxalate		Na3-citrate			Na2-tartrate		
	0.1N	0.5N	1.0N	0.1N	0.5N	0.1N	0.5N	1.0N	0.1N	0.5N	1.0N
Early non-glutinous	11.07	11.48	[illegible].85	7.87	10.92	11.67	17.72	12.37	3.73	7.69	12.83
Late semi-non-glutinous	5.36	17.23	21.13	12.36	18.12	15.81	17.40	17.21	11.58	22.05	9.27
Yellow-silky glutinous	10.30	29.59	10.14	16.53	17.45	15.18	18.85	21.20	6.93	13.39	6.83
Average	10.91	19.75	14.71	12.30	17.50	14.15	17.99	16.87	7.43	14.38	9.65

TABLE 4. *Varietal variation in protein peptization by organic acids at 0.5N concentration*

Acid	Percent of total protein peptized in indicated variety of rice			
	Early non-glutinous	Late non-glutinous	Semi-non-glutinous	Yellow silky glutinous
Acetic	5.090	7.430	4.576	14.010
Propionic	6.204	28.280	16.160	25.530
Average mono-basic acids	5.647	17.850	10.368	19.770
Oxalic	18.920	11.393	12.595	25.675
Succinic	5.277	8.429	9.482	16.365
Average di-basic acids	12.093	9.914	11.036	21.020
Lactic	10.915	13.140	9.555	23.005
Tartaric	10.915	11.235	11.470	14.780
Citric	10.310	13.430	7.964	14.050
Average hydroxy acids	10.717	12.602	9.663	17.678

It is apparent from the preceeding tables that there is a great difference i peptizability of proteins between glutinous and non-glutinous rices. The case o yellow-silky glutinous rice is perhaps the most striking example. It showed th highest percentage of protein peptized by all the seven organic acids used at all th concentrations.

SUMMARY

The peptizing capacity or power of seven organic acids and four organic salt: for the proteins of four varieties of rice was determined using concentrations o 0.1, 0.5, 1.0 and 2.0 normal.

There is a great difference in peptizability of proteins between glutinous and non-glutinous varieties of rice.

LITERATURE CITED

1. Campbell, W. R. and Hanna. Determination of nitrogen by modified Kjeldahl method. Jour. Biol. Chem. 119 1-11. 1937.
2. Cavett, J. W. An improved micro-Kjeldahl method. Jour. Lab. Clin. Med. 17:79-80. 1931.
3. Gortner, R. A., W. F. Hoffman, and W. B. Sinclair. Physico-chemical studies on proteins. III. Proteins an lyotropic series. Colloid Sym. Monograph 5:1[illegible]-195. 1928.
4. _____. Peptization of wheat flour proteins by inorganic sal solutions. Cereal Chem. 6:1-17. 19[illegible]9.
5. Hoffman, W. F. and R. A. Gortner. Preparation and analysis of the various proteins of wheat flour. Cerea Chem. 4:221-229. 1927.
6. Mangels, G. E. and J. J. Martin. Peptization of wheat flour proteins by organic acids. Cereal Chem. 12:149 157. 1935.
7. Sinclair, W. B. and R. A. Gortner. Peptization of pure protein by inorganic salt solutions. Cereal Chem. 10 171-179. 1933.
8. Staker, E. V. and R. A. Gortner. A comparative study of the peptization of the protein complex in various seeds and grains. Jour. Phys. Chem. 25:1565-1605. 1931.

有機酸及有機鹽對於米中膠類之膠化作用

羅登義　　吳志華

有機酸對於米中膠類之膠化作用，在糯和型和米之間，有有一顯著之區別，即糯米者較高，米者較低。

有機酸液及有機鹽液之濃淡于溫度，對其膠化力高低，並無重要之影響，又 CH_3 基及 OH 根與膠化力之間，未見有一定之關係。

Effects of Cholesterol-Derived Photoproducts on the Incorporation of ^{14}C-Acetate into Human Skin Lipids

Previous studies have demonstrated that either ultraviolet (UV) or sunlight reduces the level of freely extractable human skin cholesterol in vitro[1]. The fate of the cholesterol, reflected by these reduced levels, is not fully understood. However, the instability of cholesterol in the presence of air and light is well documented. The formation of cholesterol-derived photo-oxidation products under various experimental conditions have been reported[2-4]. Preliminary studies have shown that cholesterol-derived photo-oxidation products are formed in human skin upon exposure to UV light (unpublished data).

Recently BLACK and RAUSCHKOLB[5] reported that broad spectrum light (simulated sunlight) inhibited the in vitro incorporation of 1-^{14}C-acetate into human skin sterols as well as other classes of lipids. The major site of this inhibition was shown to be activation of acetate in the formation of acetyl CoA, the precursor of lipogenesis[6]. As these phenomena, reduced cholesterol levels, formation of cholesterol-derived photo-oxidation products and inhibited lipogenesis, are the result of light irradiation upon human skin, the possibility exists that they are related. The present investigations were undertaken to determine the possible role of cholesterol-derived photo-oxidation products upon skin lipogenesis.

Materials and methods. Cholesterol-derived photo-oxidation products (UV-PP) were produced by UV-irradiation of cholesterol absorbed on thin layer plates. 2 mg of cholesterol containing a trace amount of cholesterol-4-^{14}C (sp. act. 61.4 mC/mmole) in 1.0 ml of chloroform-methanol (2:1, v/v) was applied to silica gel coated thin-layer plates. The plates were irradiated for 40 min with a Burdick QA-150 N mercury arc lamp (1.43×10^5 ergs/sec/cm^2). After irradiation the plates were developed in 1,2-dichloroethane. Polar UV-PP remaining at the origin of the chromatograms were scraped off and extracted 3 times with 5 ml of chloroform-methanol (2:1, v/v). The extracts were pooled and evaporated to dryness. The radioactivity of dry residue, representing UV-PP, was equivalent to 5% of the total radioactivity of the original cholesterol.

1 F. W. RAUSCHKOLB, G. FARRELL and J. M. KNOX, J. Invest. Derm. *49*, 632 (1967).

2 W. G. DAUBEN and P. H. PAYOT, J. Am. chem. Soc. *18*, 5057 (1956).

3 E. H. MOSHACH, N. NEIRENBERG and F. E. KENDALL, J. Am. chem. Soc. *75*, 2358 (1953).

4 O. WINTERSTEINER and S. BERSTROM, J. biol. Chem. *137*, 780 (1941).

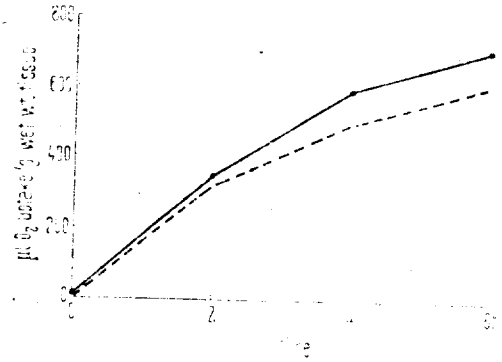
5 H. S. BLACK and F. W. RAUSCHKOLB, J. Invest. Derm. *56*, 387 (1971).

6 H. S. BLACK, J. D. SMITH, B. L. HELD and E. B. SMITH, Clin. Res. *19*, 358 (1971).

Effect of cholesterol photo-oxidation products on 1-^{14}C-acetate incorporation into human skin lipids[a]

Experimental	Total lipids	Polar[b] lipids	Fatty acids	Sterols	Triglycerides	Sterolesters
			(cpm/100 mg wet wt. tissue)			
Control	5175	3030	684	722	383	126
UV-PP	1824 (-64.9%)	1407 (-53.7%)	299 (-55.3%)	125 (-82.7%)	113 (-70.5%)	17 (-86.5%)

[a] Values represent the mean of 3 experiments. The difference between control and experimental total lipid values is significant at 1% level.
[b] Polar lipids represent phospolipids and other polar materials of total lipid extract.



Effect of cholesterol photo-oxidation products on the respiratory rates of human skin. Values represent the mean of 3 experiments. Respiratory rates are expressed as accumulative values, ●, control; ○, UV-PP treated.

Fresh human skin specimens were obtained from the lower abdomen of male Caucasians. The preparation of skin specimens has been previously described[5]. The prepared biopsies were placed in 15 ml manometric reaction flasks containing 1.0 ml of Krebs-Ringer phosphate buffer, pH 7.4, 1.0 ml tween 80 solution (56.6 mg/100 ml tween 80 in 1% ethanol), 1 µC of 1-^{14}C-acetate and 150 µg UV-PP. The concentration of UV-PP was calculated on the basis of the specific activity of radioactive cholesterol. This concentration is only approximate as radiolabelled cholesterol is known to be more susceptible to auto-oxidation than the unlabelled compound[6]. The flasks were incubated in a Gilson model GR-14 respirometer at 37°C for 6 h. After incubation, the total lipids of the specimens were extracted as previously described[5]. Radioactivity from the total lipids was determined with duplicate samples (10% of the total extract) by liquid scintillation spectrometry.

The remaining sample was reduced to approximately 50 µl under a stream of N_2. Polar lipids, fatty acids, sterols, triglycerides and sterol esters of the total lipid extract were separated on TLC plates, developed with 1, 2-dichloroethane. The different classes of lipids were visualized by the appearance of brown spots when the TLC plates were saturated with iodine vapors. The areas corresponding to the various lipid fractions were scraped from the plate directly into scintillation cocktail and radioactivity measured by liquid scintillation spectrometry.

Results and discussion. The Table shows that UV-PP actively inhibits the incorporation of 1-^{14}C-acetate into total lipids, polar lipids, fatty acids, free sterols, triglycerides and sterol esters of human skin. At least 53.7% inhibition was observed for each class of lipid. The level of inhibition reached 82.7% and 86.5% for sterols and sterol esters, respectively. The possibility that this significant inhibition might be the result of general cellular injury by UV-PP was considered. However, as seen in the Figure, the respiratory rates of UV-PP treated specimens were not greatly affected and were only 15% less than controls at the end of the experimental period.

BLACK and RATSCHKOLB[5] demonstrated that a striking reduction in 1-^{14}C-acetate incorporation into lipids occurred in skin irradiated with xenon light. In their studies only about 17% inhibition of respiratory rates of irradiated tissues occurred as compared to the 50% reduction in 1-^{14}C-acetate incorporation. In our present studies a greater inhibition of radioisotope incorporation was found, compared to their findings, with a similar effect on the respiratory rates. Although the present results could not be quantitatively correlated with their findings, the fact that the same type of inhibition occurs with both xenon light and UV-PP suggests that the effect on skin lipogenesis is mediated via similar mechanisms. Tentative identification has indicated that 5-α-cholestan-3α, 6α-diol, 7-α-OH-cholesterol, 7-β-OH-cholesterol, 7-ketocholesterol, cholesterol α-oxide and 4-cholesten-3-one are among the several constituents of UV-PP prepared in this study. These compounds have previously been reported as oxidation products of cholesterol[6,7]. Whether one constituent is responsible for the inhibitory effect of UV-PP or if the effect is a synergistic one produced by a mixture of these compounds is not known. Most, however, demonstrate structural similarities in regard to their polycyclic hydrocarbon structure. Of interest is the fact that aflatoxin B_1, a potent carcinogen, shares certain structural similarities as these oxidation products and has been shown to cause similar effects on acetate incorporation into skin lipids as those reported here for UV-PPs.

In summary, the inhibition of 1-^{14}C-acetate incorporation into skin lipids by xenon light can be replaced by UV-PP. Preliminary studies also indicate that these photo-oxidation products are, indeed, formed in UV-irradiated skin. These data suggest a possible relationship between photoproduct formation and light induced inhibition of lipogenesis. It is conceivable that photo-oxidation products formed in UV-irradiated human skin may, in turn, motivate the responsive inhibitory effects on lipogenesis[8].

Zusammenfassung. Hautbestrahlung beim Menschen mit Xenon- oder UV-Licht und die dabei beobachteten chemischen Vorgänge (Hemmung von 1-^{14}C-Azetat im Einbau in die Gesamtlipide) legen eine mögliche Relation zwischen Photoproduktbildung und lichtinduzierter Hemmung der Lipogenese nahe.

WAN-PANG LO and H. S. BLACK

Departments of Dermatology and Biochemistry, Baylor College of Medicine, and Veterans Administration Hospital, Houston, Texas 77025, U.S.A., 10 June 1971.

[7] L. A. FOSKETT and E. SYKES, J. Acoust. Soc. 77, 221 (1971).
[8] H. S. BLACK, J. D. SOLIN, L. A. ANDERS and E. W. RATSCHKOB, Experientia 26, 577 (1970).
[9] Supported by NIH Grant AM-12 [illegible], A. Welch Foundation Grant No. Q-253 and VA [illegible] Grant No. [illegible].

KETOGENIC ACTION OF SHORT CHAIN, EVEN NUMBERED CARBON FATTY ACIDS IN CARBOHYDRATE-FED ANIMALS

By EATON M. MacKAY, ARNE N. WICK, AND CYRUS P. BARNUM

(From the Scripps Metabolic Clinic, La Jolla, California)

(Received for publication, May 27, 1940)

All methods for the determination of acetone bodies in the blood give a value of 0.2 to 2.0 mg. per cent of total acetone in the blood of normal fed rats, dogs, rabbits, and humans (1). It is not known whether this small value is due to the presence of acetone bodies or the presence of some interfering material. When this value is measurably increased, we say there is a ketosis. Such an increase in the level of acetone bodies in the blood has been assumed to be dependent upon a lack of oxidizable carbohydrate such as exists during fasting and in phlorhizin or pancreatic diabetes. Heretofore such a ketosis has not been believed to occur unless the liver glycogen has been greatly depleted. Under normal circumstances fasting has been assumed to be essential. Data are presented here which show that short chain, even numbered carbon fatty acids are ketogenic and produce a ketosis as measured by the acetone body level in the blood when fed to normal carbohydrate-fed rats with large amounts of glycogen in their livers.

EXPERIMENTAL

Young adult albino rats were removed from the stock diet and for 4 hours before being fed the fatty acid esters and thereafter until sacrificed were allowed access to water and cubes of sucrose *ad libitum*. In Experiment 4 they were removed from the stock diet 12 hours earlier and sucrose allowed so that the intestinal contents would be reduced and permit observations on absorption rate. Male rats were used in Experiments 1 to 3 and female rats in Experiment 4. The latter averaged 169 gm. in weight, while the male groups averaged from 180 to 195 gm. There were three

TABLE I

Influence of Feeding Fatty Acid Esters upon Blood Acetone Bodies of Carbohydrate-Fed Rats

Experiment No.	Ethyl ester fed	Dose per sq. dm. body surface	Time after feeding											
			2 hrs.		3 hrs.		4 hrs.		6 hrs.		8 hrs.	
			Liver glycogen	Blood acetone bodies	Liver glycogen	Blood acetone bodies	Liver glycogen	Blood acetone bodies	Liver glycogen	Blood acetone bodies	Liver glycogen	Blood acetone bodies
		mM	*per cent*	*mg. per cent*	*per cent*	*mg. per cent*	*per cent*	*mg. per cent*	*per cent*	*mg. per cent*	*per cent*	*mg. per cent*
1	Ethyl alcohol	2.70					5.41	0.46				
	C_4, butyrate	2.70					5.53	2.50				
	C_6, caproate	1.80					4.90	4.06				
	C_8, caprylate	1.35					3.40	4.00				
	C_{12}, laurate	0.90					4.54	0.33				
	C_{18}, oleate	0.60					4.01	0.50				
2	Ethyl alcohol	2.70	5.96	0.90			5.50	1.05	6.57	0.17	8.97	0.30
	C_4, butyrate	2.70	9.33	2.86	6.80	5.66	4.41	2.10	7.41	3.17	11.60	1.51
	C_6, caproate	1.80	8.48	7.68	5.56	6.36	7.97	6.58	5.25	5.38	11.93	3.40
	C_{12}, laurate	0.90	8.13	0.80	6.01	0.74	9.40	0.33	9.30	0.15	10.08	0.61
	C_{18}, oleate	0.60	6.42	0.59	6.96	0.65	4.10	0.35	6.59	0.21	12.05	0.21
3	Ethyl alcohol	1.35	10.30	0.70	2.93	1.40	5.46	1.34	4.82	0.99	2.68	1.08
	C_{10}, caprate	1.08	3.43	3.90	3.42	3.96	3.22	2.91			2.65	1.92
	C_{14}, myristate	0.78	5.86	0.58	3.23	1.00	4.46	0.41	4.87	0.76		
	C_{16}, palmitate	0.67	4.93	1.11	2.27	1.05	3.99	0.99	4.24	1.07		
4	Ethyl alcohol	3.00	8.45	1.36	7.44	1.03	9.54	1.08			2.55	0.47
	C_8, caprylate	3.00	9.24	5.66	18.53	11.96	7.28	13.76			7.25	0.69
	C_{10}, caprate	2.40	6.57	6.26	9.33	8.66	6.73	8.91			5.10	17.20
	C_{12}, laurate	2.00	10.58	0.95	9.41	0.89	10.20	0.93			7.21	9.21
	C_{14}, myristate	1.71	8.56	0.42	7.94	0.64	9.51	0.64			5.15	1.34
											6.32	0.99

rats in each group and all figures represent the average of the determinations made on the members of the group.

The fatty acid esters which were fed by stomach tube came from the Eastman Kodak Company with the exception of the ethyl oleate which we prepared ourselves. This ester was used in place of the stearate, because the latter is not liquid at body temperature. Acetic acid could not be fed, for the free acid and even more so the ethyl ester are too toxic in the doses necessary to produce a definite rise in the blood acetone bodies. The other fatty acids were fed as the ethyl esters, because these are so much less toxic than the free acids. The control rats were given ethyl alcohol equivalent to the dose of butyrate in Experiments 1 and 2, of caprate in Experiment 3, and of caprylate in Experiment 4. In

TABLE II

Absorption Rate of Ethyl Esters in Experiment 4

Ester fed	Per cent absorbed at end of period			
	2 hrs.	3 hrs.	4 hrs.	8 hrs.
Caprylate	39	44	65	95
Caprate	32	54	56	69
Laurate	23	33	44	70
Myristate	30	47	74	85

each case the rats which were fed the fatty acids higher than these were also fed quantities of ethyl alcohol such that an equal amount of the ethyl radical was fed to all of the rats in a given experiment. The doses of fatty acid esters were based on the assumption that all of the carbon atoms in the fatty acids may be converted to acetone bodies.

At the end of the various periods after feeding, the rats were anesthetized with sodium pentabarbital solution, administered intraperitoneally, and an oxalated blood specimen taken from the severed abdominal aorta. Acetone bodies were determined on these specimens by the method of Barnes and Wick (1). All of the results are expressed in terms of acetone. Glycogen determinations were carried out on the livers according to the method of Good, Kramer, and Somogyi (2).

Results

Group averages are presented in Table I. The data show very clearly that fatty acids with an even number of carbon atoms, from C_4 to C_{10}, may be ketogenic in the carbohydrate-fed organism with adequate liver glycogen supplies. The fatty acids from C_{12} and up are not ketogenic under the same conditions. That these results are not primarily due to differences in the rates of absorption may be seen from Table II. These data were obtained in Experiment 4 with the same method of measuring absorption that we have applied to fats (3).

DISCUSSION

The most likely explanation of the ketogenic activity of short chain (C_{10} and lower), even numbered carbon fatty acids in the presence of an excess of carbohydrate appears to us to be associated with the inability of the body to store fat containing short chain fatty acids. Under ordinary dietary conditions the body fat contains practically no fatty acid lower than C_{14} although C_{12} may be stored when present in the diet in significant amounts (4, 5). There is no appreciable storage of capric acid (C_{10}) when it is present in the diet in reasonable quantities (5) and only 15 per cent of the carcass fat is made up of this fatty acid when 50 per cent of the calories in the diet is in the form of tripcaprin (6). Caprylic (C_8) and lower acids cannot be stored at all (4, 5, 7). Although lower fatty acids may contribute to the synthesis of longer chain acids and there is evidence (7–10) that they are able to change the nature of the fatty acids in deposit fat, we believe that they are in large part converted to acetone bodies and utilized at once, because the organism is unable to store them as such. Deuel *et al.* (11) have offered a similar explanation for the failure of a fat composed of long chain fatty acids such as cottonseed oil to give rise to glycogen, while tributyrin in equivalent amounts causes an appreciable deposition of hepatic glycogen. They suggested that since butyric acid cannot be stored in the body it is utilized and the glycerol of the tributyrin is free to be converted to glycogen. The glycerol esters of oleic, palmitic, and stearic acids of which cottonseed oil is chiefly composed on the other hand are normal components of storage fat of rats, can be

deposited as such, and the glycerol is not free to be converted to glycogen.

That the ketogenic activity of short chain fatty acids in the presence of carbohydrate is not more evident in normal blood acetone values is probably due to the very small amount of these lower fatty acids in the ordinary diet of man and animals.

The observations here reported raise the possibility that the "normal" blood acetone body levels found with all methods (1) and which persist even on a diet of pure carbohydrate are actually due to acetone bodies and result from protein catabolism. The ketogenic amino acids may be oxidized in a similar manner to their lower fatty acid counterparts.

SUMMARY

Capric acid and other fatty acids with an even number of carbon atoms and with shorter chains are ketogenic even when adequate amounts of carbohydrate are fed. The blood acetone body level is significantly raised when the ethyl esters of these fatty acids (C_4 to C_{10}) are fed to rats with liver glycogen levels of 4 per cent and higher. Fatty acids of C_{12} to C_{18} are not ketogenic.

It is suggested that the lower fatty acids are ketogenic in the presence of large amounts of liver glycogen, because unlike the longer chain acids they cannot be stored as body fat and hence are oxidized at once.

BIBLIOGRAPHY

1. Barnes, R. H., and Wick, A. N., *J. Biol. Chem.*, **131**, 413 (1939).
2. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
3. Barnes, R. H., Wick, A. N., Miller, E. S., and MacKay, E. M., *Proc. Soc. Exp. Biol. and Med.*, **40**, 651 (1939).
4. Powell, M., *J. Biol. Chem.*, **89**, 547 (1930).
5. Channon, H. J., Jenkins, G. N., and Smith, J. A. B., *Biochem. J.*, **31**, 41 (1937).
6. Powell, M., *J. Biol. Chem.*, **95**, 43 (1932).
7. Eckstein, H. C., *J. Biol. Chem.*, **81**, 613 (1929).
8. Eckstein, H. C., *Proc. Soc. Exp. Biol. and Med.*, **27**, 419 (1930).
9. Eckstein, H. C., *J. Biol. Chem.*, **84**, 353 (1929).
10. Davis, R. E., *J. Biol. Chem.*, **88**, 67 (1930).
11. Deuel, H. J., Jr., Butts, J. S., Hallman, L. F., and Cutler, C. H., *J. Biol. Chem.*, **112**, 15 (1935-36).

J. Biol. Chem. 135 (1); 157

KETOGENIC ACTIVITY OF ACETIC ACID

By EATON M. MacKAY, RICHARD H. BARNES, HERBERT O. CARNE, and ARNE N. WICK

(From the Scripps Metabolic Clinic, La Jolla, California)

(Received for publication, February 9, 1940)

It is well known (1–5) that acetic acid is rapidly utilized when fed to mammals. Whether it is oxidized directly or after conversion into other compounds is uncertain. Loeb (6) and Friedmann (7) have shown that acetic acid increases the yield of acetoacetic acid in the perfused isolated liver. The present study was designed to determine whether or not acetic acid is ketogenic in the intact organism.

The macro-Kjeldahl procedure was used for urine nitrogen determinations, Van Slyke's method (8) for the determination of urine acetone bodies, and the method of Barnes and Wick (9) for the determination of blood acetone bodies. Other methods are those which we have used before (10–12) in related studies.

After many unsuccessful trials a satisfactory experiment was carried out in which acetic acid was fed to a phlorhizinized dog. As Deuel and Milhorat (5) have noted, vomiting occurs as a usual thing after the administration of sodium acetate to dogs. Free acetic acid is even worse in this regard and only in this one experiment was emesis avoided. The cathartic action of the salt (5) is not shared by the free acid. The results in Table I show that when acetic acid is fed there is a striking increase in the excretion of acetone bodies in the urine. This was accompanied by a marked rise in the urine nitrogen excretion.

When acetic acid is fed to fasting rats (Table II), there is always an increase in the ketonuria. This is true whether the acetic acid is fed as such or partially or entirely neutralized. Blood acetone body determinations indicate that this increased urinary excretion is not a result of changes in the renal threshold but results from an increased production of acetone bodies. That

be converted to acetoacetic acid by condensation. It is known (14) that in the form of its ester acetic acid may be condensed in the test-tube to the ester of acetoacetic acid.

The increase in nitrogen excretion produced in the phlorhizinized dog by feeding acetic acid occurs to a less extent in the fasting rat. It cannot be the result of an acidosis as is the increased protein catabolism produced by feeding inorganic acids, for acetic acid does not reduce the carbon dioxide-combining power of the blood (4). The effect of acetic acid on nitrogen metabolism may not be specific, but a property of other fatty acids as well. Data on this point are not available.

This demonstration that acetic acid may give rise to acetone bodies in the intact mammalian organism has an important bearing upon the current conception of the method by which fatty acids are oxidized in the organism under circumstances such that acetone bodies are being formed. Acetone bodies are produced in the liver and carried by the blood stream to the muscles and other tissues for final oxidation. The old conception of β oxidation with the production of only 1 molecule of acetone body from 1 molecule of even the long chain fatty acids is no longer tenable. On this basis an impossible amount of fat would have to be burned to furnish the quantity of acetone bodies which is known to be utilized during a ketosis as a result of studies (15) of the difference in the concentration of acetone bodies in the arterial and venous blood. In a study of the oxidation of fatty acids by isolated liver slices Jowett and Quastel (16) found a larger quantity of acetone bodies than successive β oxidation as usually considered would account for. Then Butts (17) and Deuel (18) and their coworkers observed in rat feeding experiments a far greater yield of acetone bodies from the higher fatty acids than Knoop's hypothesis would provide for. Blixenkrone-Møller (19) compared the oxygen consumption of perfused livers of diabetic cats with the total acetone body production and could explain the low oxygen-acetone body ratio only by assuming that 4 molecules of acetone body were formed per molecule of fatty acid. Recently Stadie, Zäpp, and Lukens (20) have found that the molecular ratio of oxygen consumed to acetone bodies produced from fatty acids by liver slices is far from that which would be expected by classical β oxidation. These observations have led to the rise of

a theory for the production of acetone bodies by a mechanism other than β oxidation. Hurtley (21) first proposed this hypothesis of fatty acid oxidation in which the fatty acid is attacked at alternate carbon atoms simultaneously along the whole length of the chain. He rejected the old β oxidation hypothesis because he felt that if it were correct large amounts of the lower fatty acids intermediate between the long chain acids and the acetone bodies should be found in an intense ketosis. He overlooked the probability that these intermediate reaction compounds need only exist momentarily within the liver cells. Jowett and Quastel (16) adopted Hurtley's scheme of fatty acid oxidation which they called the "multiple alternate oxidation" hypothesis. Deuel *et al.* (18) propose a combination of the "multiple alternate oxidation" theory for the higher fatty acids with the usual β oxidation theory applied to caproic and butyric acids.

There is no direct proof of any kind to support the "multiple alternate oxidation" hypothesis as the method by which acetone bodies arise during the oxidation of fatty acids. There is plenty of evidence for the successive β oxidation of various compounds in metabolism (22). It is the most reasonable explanation of the formation of acetone bodies from caproic and butyric acids. With the evidence which has been presented in the experimental portion of this paper that acetic acid is an acetone body former the classical β oxidation hypothesis may be modified so that it may explain all of the findings which have been cited against it.

When a fatty acid is oxidized by β oxidation, 2 carbon atoms are dropped from the chain together. What form this cleavage takes is unknown but it is usually pictured as productive of acetic acid. If this were true and the acetic acid formed acetone bodies which we know it may do, there would be little difficulty in retaining the β oxidation hypothesis in the light of our present knowledge. All of the carbon atoms in a fatty acid molecule with an even number of carbon atoms could yield acetone bodies (1 molecule of $C_{16} \rightarrow 4$ molecules of acetoacetic acid; 2 molecules of $C_{18} \rightarrow 9$ molecules of acetoacetic acid; etc.) which would account for the high acetone body production which made the mechanism of β oxidation appear impossible (15, 17–19). Stadie *et al.* (20) support the multiple alternate oxidation hypothesis, because they find an observed molecular ratio of oxygen consumption to ketone

bodies produced in liver slices of 1.68:1 which is reasonably corrected to 1.51:1. They point out that this is not far from the theoretical ratio for palmitic acid, if submitted to multiple alternate oxidation, of 1.25:1. But it is much closer to the theoretical ratio of the modified β oxidation hypothesis which is 1.625:1. However, we must remember that rate observations of this kind, based upon mutilated bits of liver tissue in an abnormal environment, must be used with great care in drawing any quantitative conclusions. Because there was some unidentified fixed acid formed by liver slices during the oxidation of higher fatty acids, Jowett and Quastel (16) considered the possibility of acetone bodies being formed by β oxidation via acetic acid and discarded the idea because acetic acid produced acetone bodies at a slower rate than the higher fatty acids and because benzoate poisoning inhibited acetone body production by acetic (and butyric) acid but enhanced it for the higher fatty acids. Even though these observations had not been obtained from injured pieces of liver tissue which it is hard to believe may function normally, it would not appear necessary to discard the modified β oxidation hypothesis because of them.

The β oxidation hypothesis in its modified form should be retained until data are available which give better support of some other scheme. If it is correct, the acetic acid may never exist as such in appreciable amounts. For conditions under which extracellular (as far as the liver cells are concerned) acetone bodies are not required by the organism, that is, there is no ketosis, there is not even a need to suppose that the acetic acid is converted to acetone bodies. If β oxidation is the mechanism for the oxidation of fat when extracellular acetone bodies are not being formed, the acetic acid might be oxidized directly to carbon dioxide and water.

SUMMARY

When acetic acid is fed to a phlorhizinized dog or to fasting rats, there is an increase in the excretion of acetone bodies in the urine. Blood acetone body concentrations show that this is not due to changes in the renal threshold and the conclusion is reached that there is an increased production of acetone bodies. This is interpreted as a result of the conversion of acetic acid to acetoacetic acid.

Acetic acid feeding during fasting results in an increase in protein catabolism.

The formation of acetone bodies by acetic acid is used to support a modified view of the β oxidation hypothesis of fat metabolism which meets all of the important objections which have been raised to this hypothesis.

BIBLIOGRAPHY

1. Ringer, A. I., and Lusk, G., *Z. physiol. Chem.*, **66**, 106 (1910).
2. Lamb, A. R., and Evvard, J. M., *J. Biol. Chem.*, **37**, 329 (1919).
3. Lusk, G., *J. Biol. Chem.*, **49**, 453 (1921).
4. Taistra, S. A., *J. Biol. Chem.*, **49**, 479 (1921).
5. Deuel, H. J., Jr., and Milhorat, A. T., *J. Biol. Chem.*, **78**, 299 (1928).
6. Loeb, A., *Biochem. Z.*, **47**, 118 (1912).
7. Friedmann, E., *Biochem. Z.*, **55**, 436 (1913).
8. Van Slyke, D. D., *J. Biol. Chem.*, **32**, 455 (1917).
9. Barnes, R. H., and Wick, A. N., *J. Biol. Chem.*, **131**, 413 (1939).
10. MacKay, E. M., and Barnes, R. H., *Proc. Soc. Exp. Biol. and Med.*, **38**, 418 (1938).
11. MacKay, E. M., Sherrill, J. W., and Barnes, R. H., *J. Clin. Inv.*, **18**, 301 (1939).
12. MacKay, E. M., Wick, A. N., and Carne, H. O., *J. Biol. Chem.*, **132**, 613 (1940).
13. Lusk, G., The elements of the science of nutrition, Philadelphia, 4th edition, 399 (1928).
14. Cox, R. F. B., Kroeker, E. H., and McElvain, S. M., *J. Am. Chem. Soc.*, **56**, 1173 (1934).
15. Barnes, R. H., and Drury, D. R., *Proc. Soc. Exp. Biol. and Med.*, **36**, 350 (1937).
16. Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2159 (1935).
17. Butts, J. S., Cutler, C. H., Hallman, L., and Deuel, H. J., Jr., *J. Biol. Chem.*, **109**, 597 (1935).
18. Deuel, H. J., Jr., Hallman, L. F., Butts, J. S., and Murray, S., *J. Biol. Chem.*, **116**, 621 (1936).
19. Blixenkrone-Møller, N., *Z. physiol. Chem.*, **252**, 169 (1938).
20. Stadie, W. C., Zapp, J. A., Jr., and Lukens, F. D. W., *J. Biol. Chem.*, **132**, 423 (1940).
21. Hurtley, W. H., *Quart. J. Med.*, **9**, 301 (1916).
22. Dakin, H. D., Oxidations and reductions in the animal body, Monographs on biochemistry, London, 2nd edition (1922).

THE NUCLEUS

Vol. I (1) pp. 131-150, 1958

THE EFFECT OF ACETIC ACID AND HYDROGEN PEROXIDE ON MITOTIC FREQUENCY IN ALLIUM CEPA*

By

YRJÖ MAKINEN

Department of Botany, University of Turku, Turku, Finland.

(Received on 13th May, 1958)

I. Introduction

Although, during the last few decades, experimental cytology has developed into an extensive field of research, it has mostly been confined to description and interpretation of structural changes in the chromosome mechanism. There has been relatively much less investigation, especially with plant material, into the effect of different agents on the time scale of cell division. One of the reasons is certainly the fact that plant objects are not, as a rule, especially suitable for this type of research, as compared with such favorable material as marine eggs. For instance, *Allium cepa*, which is so much used for cytological investigation, is relatively little known in this respect. There is in the literature a review on the time relations in the mitotic cycle by Hughes (1952).

The purpose of the present investigation has been a contribution to our knowledge of the mitotic frequency relations in *Allium cepa* both in the normal condition and as affected by two simple chemical agents, acetic acid and hydrogen peroxide. Because the method of making microscopic preparations by sectioning through paraffin, and scoring the mitotic frequencies from them, is laborious, the squash method was exclusively employed in this investigation. The fact that the method has been very little used in investigations like the present has also made it especially necessary to prove the reliability of the data obtained by statistical methods. The effects of the treatments in producing chromosomal aberrations will be described elsewhere.

II. Material and Methods

A. Material:

Bulbs of *Allium cepa* L. were placed on the rim of Erlen-meyer flasks in contact with tap water, the temperature of which varied from 17 to 19°C. The water was changed every day, because from the outer scales of onion bulbs tap water dissolves a yellow pigment (the amount dissolved in distilled water is minimal), which was observed to have an injurious effect on the growth of the roots. Repeated changing

* Publications from the Department of Botany, University of Turku No. 8.

of the water was also necessary in order to prevent the growth of microorganisms. The flasks were kept protected from direct sunlight. The *p*H value of tap water fluctuated during the experiments between the limits 6·5 and 6·7.

B. Methods:

The experiments were carried out according to Levan's (1949) *Allium* test. When the roots had grown about 4 cm long, within 3-7 days (the time required varies according to the season: in autumn the emergence of roots is considerably slower than in spring), the bulbs were transferred for one day to Knop's solution, which contained in addition three drops of ten per cent ferric chloride per litre of solution. The *p*H value of the solution was adjusted to 6·7 by addition of sodium hydroxide. For treatments, the growing roots were washed first in tap water and then in distilled water and thereafter transferred to glass beakers, containing either acetic acid or hydrogen peroxide or control solution; the last-mentioned was distilled water. The surface of the solutions in the beakers was kept at such a level that only the roots were immersed.

Hydrogen peroxide solutions were made by diluting Merck's "Perhydrol" (pro analysi) with distilled water until a concentration of 0·01 M was reached. The concentrations 0·001 M and 0·0001 M were obtained by further dilution of this standard solution, the concentration of which was checked by addition of excess of potassium iodide in acid solution and titration of the liberated iodine with sodium thiosulphate. The actual concentration of the solution was observed to be 0·013 M. Correspondingly, the actual concentrations of the 0·001 M and 0·0001 M solutions used were 0·0013 M and 0·00013 M. For the sake of convenience, however, the former values are used in the text. The solutions were renewed every two weeks, owing to the weak stability of hydrogen peroxide. The acetic acid solutions were prepared by diluting from 98 per cent glacial acetic acid and checking the concentrations with a Beckman pH meter.

The concentrations of hydrogen peroxide and acetic acid used in the treatments were 0·001 M and 0·0001 M. These two concentrations were chosen on the basis of preliminary experiments in which solutions between 0·1 M and 0·000001 M were used, with different times of treatment.

Knop's solution was observed to be without any detrimental effect either on the growth of the roots or on cell divisions. Distilled water causes weak disturbances in the division mechanism (Von Rosen 1954), but, on the other hand, it was not considered advisable to add acetic acid to Knop's solution, which fact again excluded the use of it in the controls. Treatments in distilled water proved that there are no discernible differences between different times of treatment, and, accordingly, five hours was selected as a suitable time for the controls. The treatments were performed in four different series; each series included one control. The temperature of the solutions during the experiments varied within the range 17-19°C. The pH of the distilled water was 5·80-5·95.

In one part of the experiments, the root tips were fixed directly after 2, 5, and 12 hours of treatment, in the other part, the rooted bulbs were transferred, after a short rinse in tap water, to Knop's solution for recovery. Recovery times of one half, one, two, and five days were used, after which the root tips were fixed. During

the five days' recovery, the Knop's solution was changed every two days. Each treatment was carried out in duplicate but these were never arranged simultaneously.

C. Preparations:

Root tips for microscopic preparations were cut about one cm long. Each preparation was made from three or four meristematic root tips. From each treatment five preparations were made. The root tips were prepared by the Feulgen squash method.

D. The analysis of preparations:

In order to facilitate the analysis of preparations, the cover glass was divided into four squares with Indian ink lines. On examination of preparations 200 cells from every square were scored; they were chosen at random among the meristematic cells, which are recognizable by their nearly quadratic (cubic) form, their relatively large, spherical nuclei, and the regular rows of cells which they form and which in general are still visible in squash preparations. From every preparation, accordingly, 800 cells were scored and from each experiment 4000 cells. The values given in the following text are means of two experiments, and represent, if not otherwise stated, 8000 cells.

Kihlman (1952) has calculated mitotic frequencies from 1600 cells. In the present material, however, a larger number of cells proved necessary. When a comparison was made between the distribution of the mitotic frequencies for every counting square in the control (exp. No. 115) and the normal frequency curve, the following values were obtained, using X^2 analysis :

scored cells	X^2	N	P
800	—	material insufficient to calculate X^2	
1600	—		
2400	—		
3200	—		
4000	—		
4800	6·20	7	0·50
5600	3·28	7	0·80
6400	3·36	8	0·90
7200	2·54	8	0·95
8000	1·86	8	0·98

N=degrees of freedom, P= probability that the difference is due to chance. Although the material for this kind of comparison is quite small, the analyses disclose that 8000 cell scorings is the first amount to be considered fairly reliable statistically. E.g., 5600 and 6400 scorings give only suggestive results.

E. Statistical methods:

In order to check the reliability of the percentages obtained for mitotic, prophase and metaphase frequencies, they were treated statistically by making analyses of variance according to the so-called hierarchic system (Bonnier and Tedin 1940). The percentages calculated from each preparation functioned as the

basis of the analysis. The probability that the variance is due to chance alone (P) has been expressed with the following symbols:

0	0·20>	P	>	0·05
*	0·05>	P	>	0·01
**	0·01>	P	>	0·001
***	0·001	P		

The expression "mitotic frequency" has been used in the following text to indicate the proportion of dividing cells as a percentage of the total number of cells in the meristem zone. The abbreviations mean, e.g.: 0·0001 M acet./2 h/5 d=a treatment of 2 hours in 0·0001 M acetic acid, and, thereafter, a recovery time of 5 days.

III. The Normal Mitotic Frequency in Allium Cepa

The results of the control experiments, arranged in order to obtain information regarding the normal mitotic frequency in the material, are presented in table I.

Table I

The normal mitotic frequency in onion

exp.	date	mitosis	prophase		metaphase		anaphase		telophase		M/P
83	18.10.54	6·33	3·17	50·1	1·30	20·5	0·73	11·5	1·13	17·5	0·41
84	3.11.54	6·50	3·18	48·9	1·25	19·2	0·68	10·5	1·40	21·5	0·39
116	19. 1.55	6·53	3·23	49·5	1·40	21·4	0·60	9.2	1·30	19·9	0·43
115	4. 3.55	6·66	3·33	50·0	1·21	18·2	0·61	9·2	1·51	22·7	0·36

In the first column under prophase etc. are given the absolute, in the second column the relative frequencies.

The table shows that the observations made at different seasons do not deviate significantly from each other. A slight variation in the mitotic frequency percentages, however, may be observed from the average 6·44 per cent in the middle of October to 6·66 per cent in the beginning of March. The analysis of variance showed that the probability that this difference is due to chance is greater than 0·2 and, consequently, the difference cannot be attributed to any definite biological factor. It must be borne in mind, however, that the water content of onion bulbs diminishes continuously during storage and this may be the reason for the acceleration of the initiation and longitudinal growth of roots. The possible relationship between the decrease in the water content of the bulbs, the accelerated growth of the roots, and the possible increase in mitotic frequency cannot be detected on the basis of the present experiments, for the increase in the longitudinal growth of roots may also be due, at least in part, to the expansion of the cells in the prolongation zone behind the meristem. Accelerated longitudinal growth of roots in

spring is especially evident in young roots, while their length is still under one centimetre. It seems obvious that their growth has already become so uniform at a length of four cms that there is no possibility of greater differences in mitotic frequencies than table I indicates. Between the relative frequencies of the different mitotic stages there are no detectable "seasonal effects".

It can be observed in table I that about half the dividing cells are in prophase. The proportion of metaphases and telophases is about equal, i.e., *ca.* one fifth of the total, and that of the anaphases least, i.e., one tenth of all dividing cells.

Table II

The mitotic frequencies of Allium, Vicia, and Pisum

Author	plant	t	mitosis		prophase		metaphase		anaphase		telophase		M/P
exp. 115	Allium cepa	17-19°	6·66	3·33	50·0	1·21	18·2	0·61	9·2	1·51	22·7		0·36
LAUGHLIN 1919	Allium cepa	20°	—	—	85·4	—	7·8	—	2·9	—	4·6		0·09
GRAY & SCHOLES 1951	Vicia faba	19°	—	—	58·1	—	20·0	—	—	—	21·9*		0·34
KIHLMAN 1952	Pisum sativ.	19°	6·51	4·13	63·5	1·00	15·4	0·38	5·8	1·00	15·4		0·24
BROWN 1951	Pisum sativ.	20°	—	—	70·9	—	13·1	—	3·8	—	12·0		0·18

In the first column under prophase etc., are given the absolute, in the second column the relative frequencies * included anaphases.

Table II has been prepared in order to make a comparison between the present results and the earlier results of corresponding studies on different angiospermous roots recorded in the literature. Among the records presented in the table, those of Kihlman and the present author are based on the directly calculated number of dividing cells, the others on indirect methods. Hence, only the first-mentioned values could be included in the first columns of table II. The comparison of the values indicates that these—except for those published by Laughlin—are in fairly good accordance with each other. It shows that the mitotic timing system is very similar even in such separate angiospermous families as the *Liliaceae* and *Papilionaceae*. The prophase frequency in *Allium* seems to be lower, and the frequency of anaphases and telophases correspondingly higher than in *Vicia* or *Pisum*. The discrepancy of Laughlin's values becomes most apparent when the calculated relationships between metaphases and prophases (M/P) is examined. By Laughlin this ratio is exactly four times smaller than the value obtained by the present author. On the other hand, the ratio calculated from the data of Gray and Scholes is in good accordance with that of the present author.

IV. The Frequency and Duration of the Stages of the Mitotic Cycle

The duration of the stages of the mitotic cycle relative to the total duration of mitosis is the same as the ratio of their frequencies. Accordingly, on the basis of the scorings made, the duration of prophase is about half the time required for complete mitotic division, etc. The absolute duration of mitosis cannot, of course, be determined by this method. The information in the literature as to the absolute duration of mitosis varies considerably. Bersa (1927)—it is true, with great reservation—came to the conclusion that the duration of mitosis in *Zea mays* is 14·3 minutes. Stålfelt (1921) has estimated it at 12 minutes in *Pisum*, and Brown (1951) at 110 minutes in the same species. Using *Vicia faba*, Gray and Scholes (1951) have obtained the value 155 minutes. Marshak (1937) mentions that in *Vicia faba*, *Pisum sativum* and *Allium cepa*, the duration of a mitosis from the beginning of prophase to the end of anaphase is between 2·5 and 3 hours. The time found in root tips of *Allium cepa* by Laughlin (1919) is 83 minutes and by Kodani (1918) four hours.

More complicated than the relationship between the different mitotic stages is that between interphase and mitosis during a complete mitotic cycle. This is due to the fact that one cell in mitosis always gives rise to two interphase cells (cfr. Hughes 1952). Hughes has presented the following formula, calculated by Crick, for determination of the duration of an interphase:

$$\frac{\text{duration of mitosis}}{\text{duration of mitotic cycle}} \ln 2 = \ln \frac{1+2R}{1+R}$$

where R is the ratio of the cells in mitosis to all the cells of the meristem, and $\ln = \log_e$. If the intermitotic time is comparatively long, and R thus small, the right-hand side of the equation approaches R, and hence

$$\frac{\text{duration of mitosis}}{\text{duration of mitotic cycle}} = \frac{R}{\ln 2} = 1{\cdot}44\ R$$

On the basis of the results obtained in this investigation (table I, exp. 115), the value for R is 6·66/100, and hence

$$\frac{\text{duration of mitosis}}{\text{duration of mitotic cycle}} = \frac{1{\cdot}44 \cdot 6{\cdot}66}{100} = 6{\cdot}10, \text{ and}$$

duration of mitotic cycle = 10· duration of mitosis

If we take for the duration of *Allium* mitosis Kodani's (l.c.) value of four hours, the total duration of the mitotic cycle in *Allium cepa* is, accordingly, 40 hrs and the duration of the interphase 36 hrs. If, again, we take for the basis of calculations Laughlin's (l.c.) value of 83 minutes, the duration of a mitotic cycle will be about 14 hrs and the duration of the interphase about 12 hrs 30 minutes. The values

presented by Laughlin himself for the duration of a mitotic cycle are about 2 hrs and 4 hrs 30 minutes; the former was obtained at a temperature of 30° C and the latter at 10°C. These time values for the mitotic cycle are much too small, compared with the previous results from the calculations made according to Cricks' formula. It is obvious that the error in Laughlin's results arises from his method which assumed the rhythmic division of cells in root tips.

The previously presented values 14 and 40 hours are the approximate limits for the duration of the mitotic cycle within onion root tips. There is very little information concerning this duration in the literature. For instance, according to Gray and Scholes (1951) the shortest mitotic cycle in *Vicia faba* roots is 16 hrs 50 minutes and as such in good accordance with the present results. Fell and Hughes (1949) have demonstrated that in a tissue culture with mice spleen the duration of the interphase varied, according to 17 recorded cases, between 8 and 18 hours. Taking into consideration the small amount of material, this variation is fairly wide. From this point of view, it seems not improbable that the duration of the mitotic cycle may vary even in the meristem of one and the same root tip, between the previous given limits, viz., between 14 and 40 hours.

V. Factors Influencing Mitotic Frequency

Examination of the control slides revealed that the normal mitotic frequency is highly dependent on the influence of several factors. Among them may be men-

Table III

Variation in the mitotic frequency in different roots of onion

slide	frequency		slide	frequency	
65 a 1	4·83	5·75	66 a 1	4·63	4·83
65 a 2	7·63	5·50	66 a 2	7·63	4·75
65 a 3	6·50	6·00	66 a 3	6·25	5·25
65 a 4	6·00	6·75	66 a 4	4·83	7·50
65 a 5	7·00	7·13	66 b 1	8·63	7·75
65 a 6	8·50	6·33	66 b 2	6·13	9·00
65 a 7	6·75	7·63	66 c 1	7.83	8·50
65 a 8	7·00	6·83	66 c 2	8·00	7·50
65 b 1	7·50	6·63	66 c 3	6·13	8·25
65 b 2	6·75	5·25	66 c 4	6·25	6·50

Every figure in the table represents 800 cells. In the same horizontal row are the results from two root tips on the same slide. The horizontal lines separate the preparations made from different bulbs. Experiment 65 was performed on 20.8.54 and experiment 66 on 14.8.54.

tioned such generally known, more or less "normal", factors as temperature, the quality of the surrounding solution, and the variation of its pH value (cfr. Tischler 1951 pp. 1-60). The mitotic frequency is also influenced, however, by many intrinsic factors, such as the general physiological state of the roots. Not only is this statement valid regarding the roots of different bulbs but considerable variation is also to be observed in mitotic frequency between the roots of one and the same bulb (table III). The analysis of variance showed that the probability that the variation between different bulbs was due to chance is considerably greater than 0·2. Hence, it is not possible to attribute this variation between the bulbs to any definite biological factor.

In the treatments with acetic acid and hydrogen peroxide still more the physiological state of individual root has effect on the mitotic frequency than in normal conditions. The individual reaction of onion roots to treatments has also been observed by Therman (1951), Doxey and Rhodes (1949), and Bauch (1948), among others.

According to Laughlin (1919), a clear periodicity can be observed in the division of the cells in the onion root meristem. The same observation has also been made by Stålfelt (1921) in *Pisum* and by Friesner (1920) in the roots of several angiosperms. Friesner (*l.c.*) has pointed out that the periodicity recorded in his investigations is not controlled by diurnal rhythm but exclusively by the onset of germination. Laughlin (*l.c.*) has based his calculations on this same principle. By contrast, Saarnijoki (1933) has stated that the periodicity in the roots of *Brassica oleracea* depends only on diurnal rhythm and is not influenced by the onset of germination, the maximum number of divisions taking place between 9 and 11 o'clock in the morning. Nichols (1941) mentions that the maximum number of divisions in roots of germinating *Allium* seeds was observed at noon. Kellicott (1904) has made observations on the fluctuation of the mitotic cycle in Allium roots as correlated with diurnal rhythm. On the other hand, no periodicity was observed by Winter (1929) in *Gladiolus*, by Brown (1951) in *Pisum*, or by Gray and Scholes (1951) in *Vicia faba*. The records in the literature on the periodicity of the mitotic cycle are, accordingly, somewhat conflicting. In the present study, no exact analyses were made to establish the existence of periodicity but the observations made indicate that the basis of Laughlin's (1919) calculations is not correct. Evidently it is at this point that there is a source of error in his results. Although the first divisions after germination in the root tip meristem were synchronized, the periodicity will obviously be confused so rapidly that this moment is not a reliable starting point for calculations.

In order to eliminate any possible occurring diurnal fluctuation of mitotic frequency, an endeavour was made always to carry out fixation at approximate by the same time of day, i.e., they were made between 8 and 10 p.m. Since the time of fixation was kept constant, it was not possible to avoid transferring the bulbs to treatment and recovery solutions at different times of the day (when their mitotic frequency was possibly different). The error caused by this circumstance is not directly avoidable, but is indirectly by using such a large amount of material that the results are, nevertheless, statistically reliable.

Bullough (1952) has shown that the maximum frequency of divisions in the

epithelial cells of the mouse ear occurs when the animals are resting and the minimum frequency while they are active. He has also established that this variation is correlated with variations in the glucose content of the blood. It is quite possible that some diurnal changes in the composition of the cell sap may be the cause of a similar variation in *Allium*.

VI. The Effect of Treatments on the Mitotic Frequency

A. The effect of 0.001 M acetic acid :

The effect of 0·001 M acetic acid on the frequency of mitosis and its different stages is to be seen in table IV and fig. 1. The symbols + and − in the table indicate either an increase or decrease in the percentages as compared with the normal values.

Table IV

The effect of 0·001 *M acetic acid on the mitotic frequency*

treatment hrs.	recovery days	mitosis		prophase		metaphase		anaphase		telophase
0	0	6·66	3·33	50·0	1·21	18·2	0·61	9·2	1·51	22·6
	0	−4·65	−1·63	−35·0	+2·30	+49·5	−0·36	− 7·8	−0·36	−7·8
	½	−1·40	−0·81	+58·0	−0·31	+22·3	−0·09	− 6·3	−0·19	−13·4
2	1	−1·45	−0·95	+65·5	−0·30	+20·7	−0·05	− 3·4	−0·15	−10·3
	2	−3·24	−1·35	−41·7	−1·00	+30·9	−0·36	+11·6	−0·53	−16·2
	5*	−1·27	−0·44	−34·5	−0·32	+26·1	−0·18	+14·5	−0·33	+26·2
	0	−2·04	−0·68	−33·1	−1·16	+57·1	−0·11	− 5·5	−0·09	− 4·3
	½	−1·68	−0·50	−29·9	−1·00	+59·7	−0·10	− 6·0	−0·08	− 4·5
5	1	−1·15	−0·50	−44·4	−0·51	+45·6	−0·08	− 6·7	−0·04	− 3·3
	2	−4·04	−1·18	−29·1	+1·96	+48·6	−0·48	+11·8	−0·43	−10·5
	5**	−3·15	−1·81	+57·4	−0·81	+25·6	−0·31	+ 9·8	−0·23	− 7·3

In the first column under prophase etc., are given the absolute, in the second column the relative frequencies. *The figures represent 7200 **3200 cells.

As a general statement, it may be said that the mitotic frequency decreases after acetic acid treatment. After two hours' treatment, the percentages for mitotic frequency decrease, being at a minimum half a day and one day after treatment. After two days' recovery, a relatively strong increase is to be observed, after which there follows a slow decrease. The general shapes of the curves for mitosis as well as for prophase and metaphase are similar.

After five hour's treatment the decrease of mitotic frequency is greater than after two hours' treatment. The increase after the great minimum at half a day's and one day's recovery is greater, however, than after shorter treatment, and, accordingly, the percentage of mitotic frequency at the end of the experiment is higher than it is after two hours' treatment.

Although the curves in fig. 1 a, b, and c are similar in shape, the curve for metaphase frequency differs from the other two so that metaphase frequency after two hours' treatment is immediately about doubled as compared with the normal value. The cause of this phenomenon evidently lies in the arrest at metaphase, i.e., the mitosis does not progress further than the metaphase stage, while the course of the prophases continues until metaphase. The increase of the relative metaphase frequency is clearly visible in the second columns of table IV. The values are all the time above the normal one.

The results of the analyses of variance (see p. 6):

	between different treatment times	between different recovery times
mitotic frequency	*	***
prophase ,,	*	***
metaphase ,,	*	**

The part played by chance in the variation of the mitotic (either prophase and metaphase) frequency is thus greater between different treatment times than between different recovery times. This means that the frequencies of the different recovery times diverge much more than those of the different treatment times.

B. The effect of 0·0001 M acetic acid:

The effect of 0·0001 M acetic acid on the frequency of mitosis and its different stages is to be seen in table V and fig. 2. After two hours' treatment the frequency decreases, reaching a minimum after one day's recovery. After two days' recovery the percentage has attained the same value as it was immediately after the treatment but then decreases gradually towards the point of five days' recovery. The shape of the curve is, consequently, much the same as after treatment with 0·001 M acetic acid.

The relationship of the five hour curve to the two hour curve is also very similar to that in the previous treatment. Immediately after the treatment the percentage is lower than after two hours' treatment. It then decreases towards the points of half and one day's recovery but keeps below the two hour values. At the point of two days' recovery it crosses the curve for two hours' treatment and almost approaches the normal value. There is a decrease towards the point of five days' recovery but the percentages remain above the corresponding values for two hours' treatment.

The curve for twelve hours' treatment deviates from the previous ones. The percentage for mitotic frequency decreases fairly evenly, beginning from the value of two and a half per cent after treatment until one per cent at the point of two days' recovery is reached, whereafter it remains at the same level.

Table V

The effect of 0·0001 M acetic acid on the mitotic frequency

treatment hrs.	recovery days	mitosis	prophase		metaphase		anaphase		telophase	
0	0	6·66	3·33	50·0	1·21	18·2	0·61	9·2	1·51	22·6
	0	−4·45	−1·53	−34·3	+1·56	+35·1	−0·60	+13·5	−0·76	−17·1
	½	−3·29	−1·30	−39·5	+1·23	+37·3	−0·39	+11·8	−0·38	−11·4
2	1	−1·25	−0·88	+70·0	−0·29	+23·0	−0·01	− 1·0	−0·08	− 6·0
	2	−4·38	−1·80	−41·1	+1·65	+37·7	−0·58	+13·1	−0·35	− 8·0
	5	−1·50	−1·23	+75·0	−0·25	−16·7	−0·00	− 0·0	−0·13	− 8·3
	0	−2·00	−1·13	+56·3	−0·25	−12·5	−0·50	+25·0	−0·13	− 6·3
	½	−0·13	−0·13	+100·0	−0·00	− 0·0	−0·00	− 0·0	−0·00	− 0·0
5	1	−0·75	−0·00	− 0·0	−0·50	+66·7	−0·25	+33·3	−0·00	− 0·0
	2	−5·88	+4·50	+76·6	−1·13	+19·2	−0·13	− 2·1	−0·13	− 2·1
	5	−4·00	−2·13	+53·1	−0·88	+21·9	−0·50	+12·5	−0·50	−12·5
	0	−2·50	−1·38	+57·7	−0·50	−15·4	−0·13	− 3·9	−0·25	+23·1
	½	−2·25	−0·68	−33·3	−0·63	+40·7	−0·39	+14·8	−0·38	−11·1
12	1	−2·00	−0·50	−34·8	−0·63	+21·7	−0·50	+30·4	−0·38	−13·0
	2	−1·00	−0·25	−25·0	−0·63	+62·5	−0·13	+12·5	−0·00	− 0·0
	5	−1·13	−0·38	−33·3	−0·63	+55·6	−0·13	+11·1	−0·00	− 0·0

The shape of the curves for prophase frequency is quite similar as that for mitosis in general. As in the treatment with 0.001 M acetic acid, the metaphase frequency is greater than normal.

The results of the analyses of variance:

	between different treatment times	between different recovery times
mitotic frequency	*	***
prophase ,,	*	***
metaphase ,,	*	**

Again, the recovery times differ from each other much more clearly than the treatment times.

C. The effect of 0.001 M hydrogen peroxide:

The effect of 0·001 M hydrogen peroxide upon the mitotic frequency is to be seen in table VI and fig. 3. After two hours' treatment the mitotic frequency is

decreased by two and a half per cent. During a recovery period of 12 hours, the mitotic frequency decreases fairly suddenly but thereafter increases continuously and reaches almost the normal level after five days. The same is true regarding the frequency of prophase and metaphase stages. The latter frequency has even risen above the normal level.

Table VI

The effect of 0·001 *M hydrogen peroxide on the mitotic frequency*

treatment hrs.	recovery days	mitosis	prophase		metaphase		anaphase		telophase	
0	0	6·66	3·33	50·0	1·21	18·2	0·61	9·2	1·51	22·6
	0	−3·88	−1·25	−32·3	−1·00	+25·8	+1·00	+25·8	−0·63	−16·1
	½	−1·13	−0·63	+55·6	−0·38	+33·3	−0·00	− 0·0	−0·13	−11·1
2	1	−2·00	−0·75	−37·5	−0·63	+31·3	−0·38	+18·3	−0·25	−12·5
	2	−2·99	−1·13	−37·5	−0·88	+29·2	−0·38	+12·5	−0·63	−20·8
	5	−5·63	−2·63	−46·7	+1·75	+31·1	−0·25	− 4·4	−1·00	−17·8
	0	−2·63	−1·25	−47·6	−1·00	+38·1	−0·25	+ 9·5	−0·13	− 4·8
	½	−3·25	−1·38	−42·3	−0·88	+26·9	−0·50	+15·4	−0·50	−15·4
5	1	−1·88	−0·63	−33·3	−0·38	+20·0	−0·38	+20·0	−0·50	+26·7
	2	−1·50	−0·75	+50·0	−0·38	+25·0	−0·13	+12·5	−0·25	+37·5
	5	−1·00	−0·25	−25·0	−0·25	+25·0	−0·13	+12·5	−0·38	+37·5
	0	−1·50	−1·00	+66·7	−0·25	−16·7	−0·00	− 0·0	−0·25	−16·7
	½	−1·75	−0·63	−35·7	−0·75	+42·9	−0·13	− 7·1	−0·25	−14·3
12	1	−1·00	−0·63	+62·5	−0·75	+25·0	−0·13	+12·5	−0·25	− 0·0
	2	−0·63	−0·50	+80·0	−0·13	+20·0	−0·00	− 0·0	−0·00	− 0·0
	5	−1·50	−0·25	−16·7	−1·00	+66·7	−0·13	− 8·3	−0·13	− 8·3

After five hours' treatment the mitotic frequency is less than after two hours' treatment but increases during half a day's recovery and surpasses the value recorded after two hours' treatment. Beginning from this point, the percentages decrease continously until the value of five days' recovery. The observed metaphase frequency behaves quite similarly.

The frequency of twelve hour treatment decreases suddenly during the treatment, and thereafter the curve is running quite evenly. There is in the metaphase frequency a clear increase towards the end of the curve.

The results of the analyses of variance:

	between different treatment times	between different recovery times
mitotic frequency	**	***
prophase ,,	**	***
metaphase ,,	0	**

D. The effect of 0.0001 M hydrogen peroxide:

The effect is to be seen in table VII and fig. 4. A distinct decrease may be observed in the mitotic frequency of two hours' treatment. This corresponds to the similar phenomenon after treatment with 0.001 M concentration but is only visible at the point of one day's recovery, i.e., half a day later than when a stronger solution is used. Since it is reasonable to presuppose that the observed decrease does not depend on a sudden acceleration in the course of mitosis but on a diminution of the number of dividing cells, the delay of half a day in the decrease may be attributed to the weaker effect of the 0·0001 M concentration as compared with that of the 0·001 M solution.

Table VII

The effect of 0·0001 M hydrogen peroxide on the mitotic frequency

treatment hrs.	recovery days	mitosis	prophase		metaphase		anaphase		telophase	
0	0	6·66	3·33	50·0	1·21	18·2	0·61	9·2	1·51	22·6
	0	−3·00	−0·88	−29·2	−1·00	+33·3	−0·38	+12·5	−0·75	+25·0
	½	−3·38	−0·88	−25·9	−2·00	+59·3	−0·13	− 3·7	−0·38	−11·1
2	1	−0·25	−0·13	±50·0	−0·00	− 0·0	−0·13	+50·0	−0·00	− 0·0
	2	−3·25	−1·25	−38·5	−1·75	+53·9	−0·13	− 3·9	−0·13	− 3·9
	5	−2·13	−0·63	−29·4	−1·00	+47·1	−0·25	+11·8	−0·25	−11·8
	0	−2·75	−0·50	−18·2	−1·38	+50·0	−0·75	+27·3	−0·13	− 4·6
	½	−2·50	−0·88	−35·0	−0·88	+35·0	−0·25	+10·0	−0·50	−20·0
5	1	−4·50	−1·75	−38·9	−1·25	+27·8	−0·88	+19·4	−0·63	−13·9
	2	−0·88	−0·75	+85·7	−0·13	−14·3	−0·00	− 0·0	−0·00	− 0·0
	5	−0·75	−0·38	±50·0	−0·25	+33·3	−0·00	− 0·0	−0·13	−16·7
	0	−2·13	−0·49	−23·0	−0·88	+41·3	−0·38	+17·8	−0·38	−17·8
	½	−1·50	−0·37	−24·7	−0·38	+25·5	−0·25	+16·7	−0·50	+33·3
12	1	−1·88	−0·88	−46·7	−0·63	+33·3	−0·25	+13·3	−0·13	− 6·7
	2	−0·88	−0·50	+57·1	−0·38	+42·9	−0·00	− 0·0	−0·00	− 0·0
	5	−0·50	−0·25	±50·0	−0·25	+50·0	−0·00	− 0·0	−0·00	− 0·0

After five hours' treatment a similar delay has been recorded. In the curve a distinct increase may be observed in the mitotic frequency at the point of one day's recovery. It corresponds to the increase observed at the point of half a day's recovery after five hours' treatment with 0·001 M concentration. This increase is greater than after the corresponding treatment with 0.001 M solution, having its counterpart in the greater decrease of 0.0001 M/2 hrs' treatment as compared with 0·001 M/2 hrs' treatment.

In every curve (mitotic, prophase, and metaphase frequency) after 12 hours' treatment a slight peak may be observed at the point of one day's recovery. This peak is followed by a continuous gentle decrease is the frequencies.

The curves for prophase and metaphase frequencies are in general similar to those for mitotic frequency.

The results of the analyses of variance:

	between different treatment times	between different recovery times
mitotic frequency	*	***
prophase „	*	***
metaphase „	*	*

VII. Discussion

A. General remarks:

Attempts to give a detailed interpretation of the curves and values representing the variation in the frequency of mitosis and its different stages, soon lead us into pure speculation. For example, if we examine fig. 1 or table IV we observe that the prophase frequency has fallen from the normal value of 3·33 per cent to 1·63 per cent. This phenomenon may depend on either of the following factors: (1) The course of prophase has been accelerated as compared with other stages of mitosis, or (2) the absolute frequency of the cells at prophase has decreased. Point (2) contains two different possibilities: (a) prophases have advanced to the metaphase stage whilst no new prophases have begun, or (b) a number of prophases have reverted to the interphase.

Point (2a) appears most likely, since at the same time the metaphase frequency has increased. It must be borne in mind, however, that there exists a possibility for a simultaneous effect of several of the above-mentioned factors. Accordingly, it is theoretically possible to formulate seven different combinations to interpret the above-mentioned decrease in prophase frequency. The relative effect of different partial factors may, of course, be variable.

On the basis of the aforesaid the following discussion will deal only with certain observations of a more general nature.

B. Acetic acid treatment:

On the basis of figs. 1 and 2 and tables IV and V, three generalisations may be made regarding the changes after treatments in the frequency of mitosis and its stages:

(1) The frequency of mitosis and its stages has decreased as a result of treat-

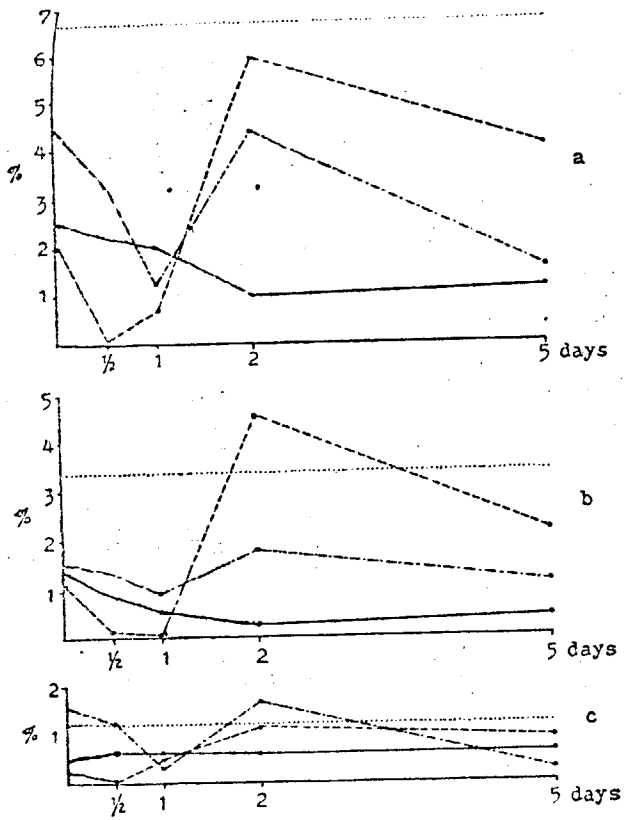


Fig. 1. The effect of 0.001 M acetic acid on the mitotic (a), prophase (b) and metaphase (c) frequency. The dotted line indicates the normal frequency. -- the treatment for two, -- five hours. On the abscissa the recovery times in days.

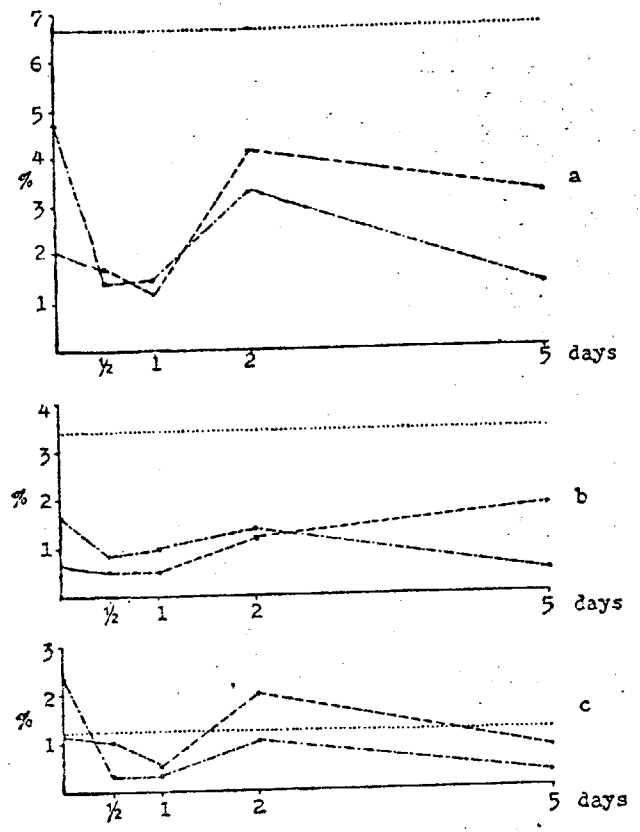


Fig. 2. The effect of 0.0001 M acetic acid on the mitotic (a), prophase (b) and metaphase (c) frequency. The dotted line indicates the normal frequency. -- the treatment for two, -- five, — twelve hours. On the abscissa the recovery times in days.

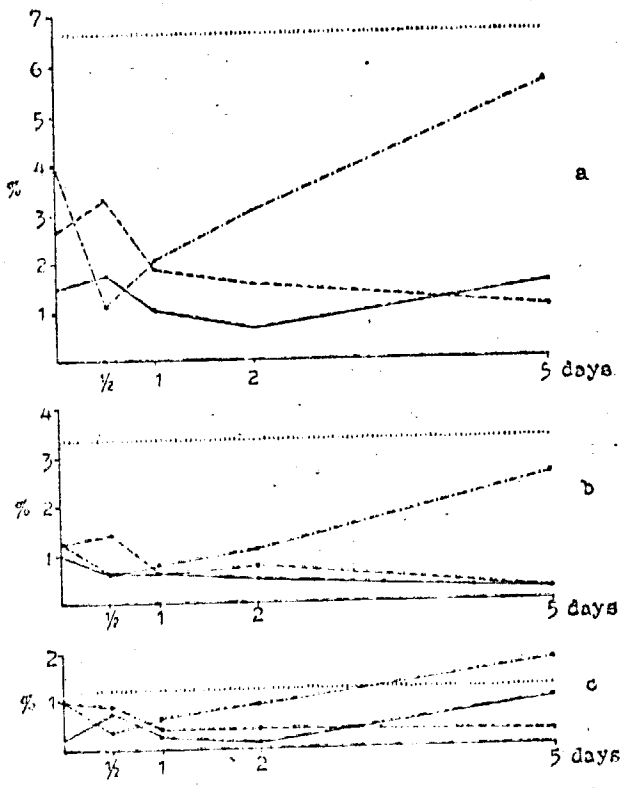


Fig. 3. The effect of 0.001 M hydrogen peroxide on the mitotic (a), prophase (b) and metaphase (c) frequency. See fig. 2 for explanation.

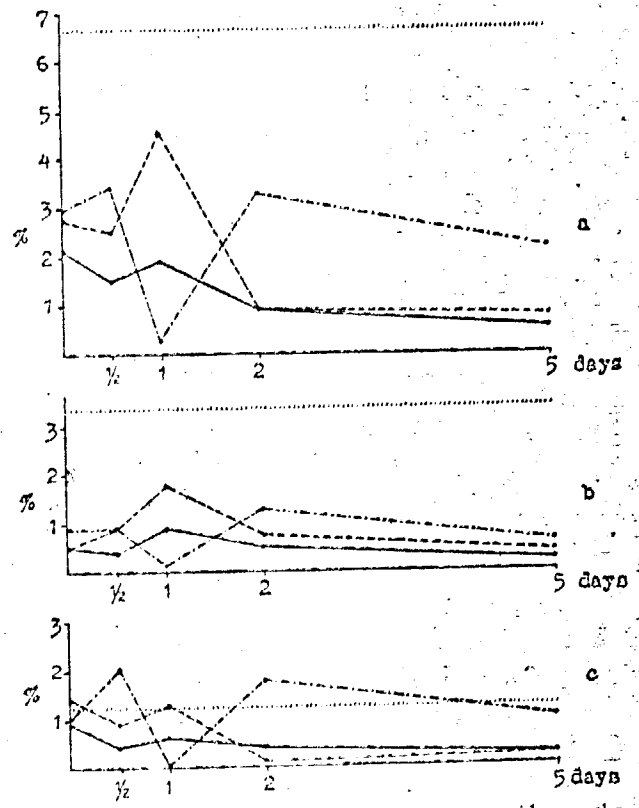


Fig. 4. The effect of 0.0001 M hydrogen peroxide on the mitotic (a), prophase (b) and metaphase (c) frequency. See fig. 2 for explanation.

ment with acetic acid. The only exception is the metaphase frequency after two hours' treatment.

(2) During the recovery the decrease of frequency continues for half a day, after which there begins an increase of the frequency which reaches a maximum after two days. A new decrease may be recorded thereafter. The prophase frequency after treatment with 0·001 M solution forms an exceeption.

(3) The frequencies remain below the normal values even during the recovery periods. The metaphases are, however, exceptional in this respect.

It was pointed out previously, in connection with the comparison between two and five hours' treatment with 0·001 M and 0·0001 M solutions, that after a recovery of one or two days the curve for frequencies after five hours' treatment surpasses, as a rule, that after two hours' treatment. This may be explained by assuming that during the treatment and the subsequent recovery period the five hours' treatment has caused a decrease in the absolute number of dividing cells (either in such a way that a smaller number of interphases has reached the prophase stage, or by increasing the number of cells that have passed from ana-or telophases into interphase). After the above-mentioned one or two days' recovery the difference in this respect between times of treatment has become equalized but now the more retarding effect on mitosis of longer treatment time appears, and the mitotic frequency is, consequently, greater. The retardation of the course of mitosis means a prolongation of its duration. If, however, the duration of interphase were to increase proportionally, it would mean that the relationship between mitosis and interphase, i.e., the mitotic frequency, would remain unchanged. It is, therefore, evident that the relative duration of interphase has become shorter.

The second columns of tables IV and V show that the relative importance of the metaphase frequency has increased. It is evident that the duration of metaphase has increased proportionally more than that of other stages of mitosis, or, in other words, on acetic acid treatment an accumulation of metaphases has taken place.

The effects of factors having an influence on mitotic frequency may be divided into three groups:

(1) The relative lengths of the mitotic stages have not changed. In *Vicia faba* Gray and Scholes (1951) have demonstrated that after irradiation with x-rays the curves representing the duration of the different mitotic stages remain more or less unchanged. Deysson (1954) also obtained negative results when he studied the effect of maleic hydrazide in changing the frequencies of the mitotic stages.

(2) The relative duration of prophase has been prolonged in contrast to that of the other stages, which has been shortened. The majority of observations in the literature refer to changes of this type. D'Amato (1950a and the literature cited there), who has especially investigated the effects of this type produced by a number of chemical compounds, distinguishes between preprophase and prophase poisoning. The former affects interphase nuclei and prevents the onset of mitosis in them, the latter prophase nuclei, which it prevents from reaching the metaphase stage. The two processes often occur simultaneously. According to D'Amato (1950a), prophase poisoning is always followed by preprophase poisoning and c-mitosis.

Other mitotic stages, beginning from metaphase, remain unchanged or their duration is only slightly prolonged. Compounds of this mode of action are, e.g., ethylene glycol (D'Amato 1948), allylic isothiocyanate (D'Amato and Avanzi 1949a), sodium dichlorphenoxyacetate (D'Amato and Avanzi 1949b), acridine (D'Amato 1950b), naphthalene acetic acid (Berger and Witkus 1949), and pyrogallol (Tjio 1951). The effects of X-ray irradiation, are as a rule, of this type.

(3) The duration of metaphases has been prolonged, that of the other stages shortened. The accumulation of metaphases is characteristic of all c-mitotic compounds, which narcotize or destroy the spindle mechanism and thereby prevent the transition of metaphases to anaphases. Changes belonging to this group are caused by acenaphthene (Schmuck 1938), arsenic compounds (Dustin 1947), sodium ribonucleate (Kodani 1948), adenylic acid (Kodani, *l.c.*), adenine (Kihlman 1952), and, of course, colchicine (Blakeslee and Avery 1937). Ostergren and Levan (1943) mention some further compounds acting in the same manner. X-ray irradiation may also have a similar effect (D'Amato 1950a).

Acetic acid should undoubtedly be included in this group. This fact becomes most apparent when the ratio between metaphases and prophases (M/P) is calculated. In table VIII are given these ratios in acetic acid and hydrogen peroxide treatments. As is seen from table I, the normal ratio varies between 0·36 and 0·43. When the values for each treatment (0·001 M, 0·0001 M acetic acid, 0·001 M, 0·0001 M hydrogen peroxide) are compared with the normal ratio, analysis of variance gave:

0·001 M acetic acid	..	*
0·0001 M acetic acid	..	0
0·001 M hydrogen peroxide	..	**
0·0001 M hydrogen peroxide	..	*

The increase of the ratio M/P is thus clear, although not very convincing. Only in 0·0001 M acetic acid treatment is it suggestive (a weaker effect as compared to 0·001 M acetic acid).

Darlington (1937), in order to clarify the different events included in meiosis, has developed his precocity theory, which means that the meiotic processes taking place outside the chromosomes are precocious as compared with those within the chromosomes. Oksala (1943, 1944) has further developed this theory, laying special stress on the precocity of the spindle. According to him, in the spermatogenesis of the dragonfly *Aeschna grandis* certain phenomena may be observed in the last premeiotic divisions which are connected with the onset of meiosis. Especially characteristic among them is the rapid increase in the numerical value which illustrates the ratio between prometaphase and late prophase.

The precocity of the spindle may be considered the most likely explanation of the accumulation of metaphases after acetic acid treatment in Allium roots, although there may also be observed such phenomena as chromosome contraction and formation of loops between chromatids, which, according to Ostergren (1944), are characteristic of c-mitosis. Acetic acid differs sharply from the c-mitotic compounds in the occurrence of precocity of spindle formation. Whereas in c-mitosis

Table VIII

The ratio M/P in acetic acid and hydrogen peroxide treatments

treatment hrs.	recovery days	M/P			
		acetic acid		hydrogen peroxide	
		0·001 M	0·0001 M	0·001 M	0·0001 M
0	0	0·36	0·36	0·36	0·36
2	0	+1·42	+1·02	+0·80	+1·14
	½	+0·38	+0·94	+0·60	+2·29
	1	−0·32	−0·33	+0·83	−0·00
	2	+0·74	+0·93	+0·78	+1·40
	5	+0·72	−0·22	+0·67	+1·60
5	0	+1·72	−0·22	+0·80	+2·75
	½	+2·00	−0·00	+0·64	+1·00
	1	+1·03	+ δ	+0·60	+0·71
	2	+1·67	−0·25	+0·50	−0·17
	5	+0·45	+0·41	+1·00	+0·67
12	0	—	−0·27	−0·25	+1·80
	½	—	+1·22	+1·20	+1·03
	1	—	+0·75	+0·40	+0·71
	2	—	+2·50	−0·25	+0·75
	5	—	+1·67	+4·00	+1·00

prevention of spindle formation leads to polyploidy, the ultimate result of spindle precocity is reduction of chromosome number.

C. Hydrogen peroxide treatment:

The curves representing mitotic frequency after hydrogen peroxide treatment differ in some respects from those obtained after acetic acid treatment.

(1) The values for mitotic frequency rise after the treatment with 0·001 M hydrogen peroxide after a recovery period of two days and their decline is only slight after treatment with 0·0001 M solution and the same recovery time (after the corresponding treatment with acetic acid the decline is more conspicuous). This demonstrates the lesser effect of hydrogen peroxide compared to that of acetic acid.

(2) When curves for two and five hours' treatment are compared with each other, it may be observed that after one half and one day's recovery the curve for five hours' treatment lies consistently above the curve for two hours' treatment, but after other recovery times below it (figs. 3 and 4). The shape of the curves

may be explained by assuming that the inhibition effect of five hours' treatment is stronger than that of two hours' treatment. When the bulbs are transferred to recovery solution, the effect of the longer treatment manifests itself, in comparison with two hours' treatment, as a greater retardation of mitosis. The normal condition is reached in roots after two hours' treatment, beginning from one day's recovery, while the effect of five hours' treatment still continues.

The curves for twelve hours' treatment are similar after both acetic acid and hydrogen peroxide, showing that the effect of both treatments has been more or less lethal.

As becomes evident on examining table VIII, the effect of hydrogen peroxide on the ratio M/P is similar to that of acetic acid.

Summary

1. The effect of acetic acid and hydrogen peroxide on the mitotic frequency of *Allium cepa* roots has been studied, after 0, 2, 5, and 12 hours' treatment with 0.001 M and 0·0001 M solutions, and subsequent 0, ½, 1, 2, and 5 days' recovery periods.

2. The fluctuation of mitotic frequency is great even in normal conditions, which makes it necessary to use ample material. The analyses of variance performed showed that the mitotic frequencies calculated from the present experimental data are sufficiently reliable.

3. The values obtained for normal mitotic frequency were between 6·33 and 6·66 per cent. The calculated value for the duration of the mitotic cycle is between 14 and 40 hours.

4. Treatments with acetic acid and hydrogen peroxide in general decrease the values for frequency of mitosis and its different stages as compared with normal conditions.

5. The relative frequency of metaphase, and consequently the ratio between metaphases and prophases (M/P) is increased by treatments with both acetic acid and hydrogen peroxide. This phenomenon is explained in terms of the precocity theory, the function of the spindle being assumed so much to precede the division of the chromosomes into functional units that anaphase separation is prevented by the retardation of the latter process. This effect is, accordingly, sharply distinct from that of the c-mitotic compounds, the effect of which is chiefly due to the prevention of spindle activity.

6. There exists a special relationship between the frequencies after two and five hours' treatment with acetic acid and hydrogen peroxide. A hypothesis to explain this phenomenon has been put forward.

7. The treatment of 12 hours' is more or less lethal.

Acknowledgement:

The author is greatly indebted to the Head of the Department of Botany, University of Turku, Dr. Antero Vaarama, for his critical advice and suggestions during the work.

REFERENCES

BAUCH, R., 1948. Irreversibele Chromosomenschädigungen durch Trypaflavin. *Planta.* **35**: 536-554

BERGER, C. A, & WITKUS, E. R., 1949. Further studies of the cytological effects of combined treatments with colchicine and naphthaleneacetic acid. *Amer. J. Bot.* **36**: 794-795.

BERSA, E., 1927. Strahlenbiologische Untersuchungen II. Über die Wirkung der Röntgenstrahlen auf die Kernteilung der Wurzelspitzen von *Zea mays. Sit.ber. Akad. Wiss. Wien. Math-naturw. Kl.*, I., **136**: 383-401.

BLAKESLEE A. F. & AVERY. A. G., 1937. Methods of inducing doubling of chromosomes in plants by treatment with colchicine. *J. Hered.* **28**: 393-411.

BONNIER, G. & TEDIN, O., 1940. Biologisk variationsanalys. 325 pp. Stockholm.

BROWA, R., 1951. The effects of temperature on the durations of the different stages of cell division in the root-tip. *J. Exp. Bot.* **2**: 96-110.

BULLOUGH, W. S., 1952. Energy relations of mitotic activity. *Biol. Rev.* **27**: 133-168.

D'AMATO, F., 1948. The effect of colchicine and ethylene glycol on sticky chromosomes in Allium cepa. *Hereditas.* **34**: 83-103.

——, 1950*a*. The quantitative study of mitotic poisons by the Allium cepa test: data and problems. *Protoplasma.* **39**: 423-433.

——, 1950*b*. Studio statistico dell' attivita mutagena dell' acridina e derivati. *Caryologia*, **2**: 229-297.

——, & AVANZI, M. G., 1949*a*. Studio comparato dell' attivitá citologica di alcune essence. *Caryologia*, **1**: 175-193.

——&——, 1949*b*. Primo contributo alla conoscenza dell' attivitá citologica e fisiologica delle sostanze di crescita sul testo Allium cepa L. *Caryologia.* **2**: 31-45.

DARLINGTON C. D., 1937. Recent advances in cytology. 2nd ed. London.

DEYSSON, G., 1954. Sur les divers types d'action antimitotique. *Comm. VIIIe Congr. Int. Bot. Paris.* Sect. 9 & 10, 13.

DOXEY, D. & RHODES, A., 1949. The effect of the plant growth-regulator 4-chloro-2-methylphenoxyacetic acid on mitosis in the onion (*Allium cepa*). *Ann. Bot.*, N. S. **13**: 105-111.

DUSTIN, P., 1947. The cytological action of ethylcarbamate (urethane) and other carbamic esters in normal and leukaemic mice, and in rabbits. *Brit. J. Cancer.* **1**: 48-59.

FELL, H. B. & HUGHES, A. F., 1949. Mitosis in the mouse: a study of living and fixed cells in tissue culture. *Quart. J. Micr. Sci.* **90**: 355-380.

FRIESNER, R. C., 1920. Daily rhythms of elongation and cell division in certain roots. *Amer. J. Bot.* **7**: 380-416.

GRAY, L. H. & SCHOLES, M. E., 1951. The effect of ionizing radiations on the broad bean root. Part VIII. Growth rate studies and histological analyses. *Brit. J. Radiol.* **24**: 82-92, 176-180, 228-236, 285-291, 348-352.

HUGHES, A., 1952. The mitotic cycle. The cytoplasm and nucleus during interphase and mitosis. 232 pp. London.

KELLICOTT, W. E., 1904. The daily periodicity of cell-division and of elongation in the root of Allium. *Bull. Torr. Bot. Club.* **31**: 529-550.

KIHLMAN, B., 1952. Induction of chromosome changes with purine derivatives. *Symb. Bot. Ups.* **XI**: 4, 1-96.

KODANI, M., 1948. Sodium ribose nucleate and mitosis. *J. Hered.* **39**: 327-335.

LAUGHLIN, H. H., 1919. Duration of the several mitotic stages in the dividing root-tip cells of the common onion. *Publ. Carn. Inst. Washington*, 265.

LEVAN, A., 1949. The influence on chromosomes and mitosis of chemicals, as studied by the Allium test. *Proc. 8th Int. Congr. Genet.* (*Hereditas.* suppl. vol.) 325-337.

MARSHAK, A., 1937. The effect of X-rays on chromosomes in mitosis. *Proc. Nat. Acad. Sci.* **23**: 362-369.

NICHOLS, C., 1941. Spontaneous chromosome aberrations in Allium. *Genetics.* **26**: 89-100.

ORSALA T. 1943. Zytologische Studien an Odonaten I. Chromosomenverhältnisse bei der Gattung Aeschna mit besonderer Berücksichtigung der postreduktionellen Teilung der Bivalente. *Ann. Acad. Sci. Fenn.* A. **IV**: 4 1-63.

——1944. Zytologische Studien an Odonaten II. Die Entstehung der meiotischen Prakozitat. *Ann. Acad. Sci. Fenn.* A. **IV**: 5 1-33.

Von Rosen, G. 1954: Breaking of chromosomes by the action of elements of the periodical system and by some other principles. *Hereditas.* **40**: 258-263.

Saarnijoki, S. 1933. Über die tägliche Periodizität der Kernteilung in den Keimwurzelspitzen von Brassica oleracea. *Ann. Bot. Soc. 'Vanamo'* **4**: 10: 1-13.

Schmuck, A. 1938. The chemical nature of substances inducing polyploidy in plants. *Compt. Rend. Acad. Sci. URSS.* N. S. **19**: 189-192.

Stalfelt M. G. 1921. Studien über die Periodizitäl der Zellteilung und sich daran anschliessende Erscheinungen. *Kungl. Sv. Vet. Handl.* **62**: 1 1-114.

Therman, E., 1951. The effect of indole-3-acetic acid on resting plant nuclei. I Allium cepa. *Ann. Acad. Sci. Fenn.*, A. **IV**: 16, 1-40.

Tischler, G., 1951. Allgemeine Pflanzenkaryologie. Kernteilung and Kernverschmelzung. Handbuch der Pflanzenanatomie. 2nd ed. Berlin.

Tjio, J. H., 1951. Chromosome fragmentation by pyrogallol in Vicia faba. *An. Aula. Dei.* **2**: 187-194.

Winter, J. M., 1929. Some observations on the rate of mitosis in root tip meristems of Gladiolus. *Trans. Amer. Micr. Soc.* **48**: 276-291.

Ostergren, G., 1944. Colchicine mitosis, chromosome contraction, narcosis and protein chain folding. *Hereditas.* **30**: 429-467.

——& Levan, A., 1943. The connection between c-mitotic action and water solubility in some monocyclic compounds. *Hereditas.* **29**: 496-498.

Mallinckrodt Chemical Works Technical Data

56% Acetic Acid

Specifications:

Acetic Acid Content % By Weight	55 - 57%
Color (APHA as is)	150 APHA max.
Appearance	Clear and no sediment

SODIUM DIACETATE
Technical

Sodium Diacetate is used in applications such as a buffer, mold inhibitor for bread and rope forming bacteria, souring agent, intermediate for acid salts, ready source of acetic acid, anti-tarnishing agent, mordant, and varnish hardener.

Chemical and Physical Specifications

Color	White
Odor	Typical Acetic
APHA, 10% Solution	Less than 10
pH, 10% Solution	4.5 - 5.0
Moisture	9% Maximum

Assay

Acetic Acid	33% - 35%
Sodium Acetate	57% - 60%
Sodium Chloride	0.18% Maximum
Fe	Less than .003%

SODIUM DIACETATE
Anhydrous

$CH_3COONa \cdot CH_3COOH$

Sodium Diacetate, Anhydrous, is used as a buffering agent, mold inhibitor for bread and rope forming bacteria, souring agent, intermediate for acid salts, anti-tarnishing agent, mordant, and varnish hardener.

Chemical Specifications

Color	White
Odor	Typical Acetic
APHA	Less than 10
pH, 10% Solution	4.5 - 5.0
Moisture	1% Maximum

Assay

Acetic Acid	39% - 41%
Sodium Acetate	57% - 60%
Sodium Chloride	0.18% Maximum
Fe	Less than .003%

Packing

250 lb. fiber drums, 55 gallon Leverpak with poly liner or 50 lb. multi-wall, poly-lined bag.

SODIUM ACETATE, ANHYDROUS

Specifications

Appearance	White Powder
Odor	Characteristic Acetic Odor
Mositure Content	0.8% Max.
pH (10% Aqueous Sol'n.)	7.0 - 9.0
Color (APHA 15% Aq. Sol'n.)	25 Max.
Assay	99.0% Min.
Free Acid (As Acetic Acid)	0.025% Max.
Free Alkali (as Na_2CO_3)	0.04% Max.
Iron Content	10 ppm Max.
Heavy Metal	10 ppm Max
Sodium Carbonate	0.1% Max.
Sodium Chloride	0.3% Max.

Screen Test

On 20 Mesh	5% Max.
On 40 Mesh	70% Max.
Thru 100 Mesh	5% Max.

Bulk Density

loose	0.68
Packed	0.79

GLACIAL ACETIC ACID

Appearance:	Clear, free from suspended matter
Color	10 Hazen Units (Max.)
Assay	99.85% W/W (min.)
Residue on Evaporation	0.005% W/W (max.)
Chlorides (as Cl)	1 ppm (max.)
Sulfates (as SO_4)	1 ppm Max.
Iron	2 ppm Max.
Wt. per ml. at 20°C	1.048 - 1.051
Water Content	0.15% W/W Max.
Distillation range at 760 mm hg.	
I.B.P.:	117.5°C Min.
F.B.P.:	118.0°C Max.
Heavy Metals (as Pb)	3 ppm (max.)
Aldehydes (as Acetaldehyde)	0.03% W/W (max.)
Permanganate Time	2 Hours (min.)
Formic Acid	0.10% W/W Max.
Sulfurous Acid	1 ppm (max.)
Crystallizing Point	16.35°C (min.)

ACETIC ACID

Acetic Acid Content, % by weight, Min.	80.0%
Freezing Point, °F Min.	17.6
Color APHA Max.	10
Water Content, % by Weight, Max.	20%
Acetaldehyde Content, % by weight Max.	0.05
Iron Content, ppm by weight, Max.	1.0
Heavy Metals Content	None by Test
Chloride Content, ppm by weight Max.	1.0
Sulfate Content, ppm by weight Max.	1.0
Sulfurous Acid Content, ppm by weight Max.	1.0
Permanganate time, min.	2 hrs.
Formic Acid Content, % by weight, Max.	0.05

ACETIC ACID

Acetic Acid Content, % by weight, min.	70.0%
Freezing Point, °F, min.	-0.5
Color, APHA max.	10
Acetaldehyde Content, % by weight, max.	0.05
Iron Content, ppm by weight, max.	1.0
Heavy Metals Content, ppm by weight, max.	None, by test
Chloride Content, ppm by weight, max.	1.0
Sulfate Content, ppm by weight, max.	1.0
Permanganate Time, min. (ACS Test)	2 hrs.
Formic Acid Content, % by weight, max.	0.05
Sulfurous Acid Content, ppm by weight, max.	1.0

THE NUCLEUS Vol. 9(2) : pp. 119-131, 1966

SPERMATOCYTE CHROMOSOME ABERRATIONS IN TWO SPECIES OF GRASSHOPPERS AT TWO DIFFERENT IONIC ACTIVITIES

By

G. K. MANNA AND PRANAB K. MUKHERJEE*
Department of Zoology, University of Kalyani, Kalyani, Nadia, West Bengal, India

(Received on 3rd March 1966)

Introduction

In an early preliminary note Manna and Ray (1964) reported that changes in the spermatocyte chromosomes of the grasshopper, *Spathosternum prasiniferum*, could be introduced by injecting acidic and alkaline solutions. Since the discovery of mutagenic action of nitrogen mustard in animals by Auerbach and Robson (1947) and urethane in plants by Oehlkers (1943), a large number of chemicals has been tested for inducing cellular damages. The effects of various chemicals used by different workers in different plant and animal materials have been ably reviewed by many workers (Auerbach, 1951; Blesele, 1958; D'Amato, 1950; Grundmann, 1961; Eigsti and Dustin, 1957; Kihlman, 1961; Sharma and Sharma, 1960; Wilson, 1960). Further, Kihlman (1956) showed that at least the effect of some of the chemical mutagens as for example, maleic hydrazide, may indirectly be connected with the hydrogen ion concentration of the treated cell. In order to understand many biological problems centering round the evolutionary conception, the study of structural changes of chromosomes is of fundamental importance. Such structural changes often have to be produced by artificial means since spontaneous ones occur very rarely. Of the two important means of inducing chromosomal changes, the ionizing radiations (see Hollaender, 1954; Bacq and Alexander, 1955) and the radiomimetic chemicals, the latter originally was thought to be of greater utility particularly for localized breaks. However, this expectation has not been realized in all cases from the actual data, although the other implications of such studies are still important. The pH dependence effect in the chromosomes opens up an interesting field of investigation. The effect of chemicals in the animal chromosomes has not been tested as widely as that of the plant materials, and in recent years a new problem has been presented by virus-associated mammalian chromosome breakage (see Aula, 1965).

Further, the response of animal chromosomes in relation to breakage or other changes through different chemical treatments appears to be relatively poor in comparison to that of plant materials. Thus Raychaudhuri (1961) suggested that this difference in response might be attributable to the difference in the metabolism of the two classes of materials. However, with the accumulation

*Acknowledgements are made to CSIR, Govt. of India for a junior fellowship awarded to PKM.

of more data on animal materials, the response between plants and animals may not appear to be so wide. In recent years more records of the chemical effect in animal materials similar to that of plants are being published (Hsu and Somers, 1961; Manna and Mazumder, 1964; Manna and Parida, 1965a, b, 1966 and unpublished; Sarkar, 1956).

The reports on the spermatocyte chromosome changes of grasshopper induced by various chemical agents are very limited. Yosida (1950), Manna and Parida (1965a) studied the effect of colchicine on the male meiotic cells treated in various ways and fixed at different hours of post-treatment period. Sarkar (1956) studied the effect of nitrogen mustard in the spermatocyte cells of a grasshopper, *Gesonda punctifrons*. Recently Manna and Mazumder (1964) have reported the specific type of break in the X-chromosome of the first meiotic prophase cells in one of the two species of grasshoppers treated with ethyl alcohol. Manna and Parida (1965b, 1966 and unpublished) are making a series of investigations on the effect of radiomimetic chemicals and mitotic poisons in the spermatocyte cells of different species of grasshoppers.

The present investigation was undertaken with a view to find out the effect of the acidic and alkaline solutions of some known pH upon the spermatocyte cells of two species of grasshoppers.

Material and Method

Two species of adult male grasshoppers, *Spathosternum prasiniferum* and *Oxya velox*, constituted materials for the present study. Specimens were injected with water, 0·67% normal saline and various kinds of solutions having the pH adjusted at some particular level as detailed below (table 1). The pH level of the solutions was tested prior to the injection. Each experiment was repeated twice or more but in the table they have been added together. The effects produced by solutions of different pH were repeatable.

The alkaline solution of pH 9 was prepared by adding sodium hydroxide to 0·67% saline solution. Each specimen of *Spathosternum prasiniferum* was injected abdominally with 0·02 ml. of the solution. In one series, acidic pH solutions of 3 and 4 were prepared by mixing hydrochloric acid with 0·67% normal saline solution. Approximately 0·02 ml. of the solution was injected into the abdomen of *S. prasiniferum*. In another series individuals of *Oxya velox* were injected with an acidic solution of pH 4 prepared by dilating acetic acid in distilled water (0·04 M acetic acid solution gives a pH of about 4).

The data (table 1) show that the mortality was higher among specimens treated with HCl than those with acetic acid solution. However, the size of *Spathosternum prasiniferum* was relatively smaller than *Oxya velox*. At present the mortality rate cannot be definitely correlated with size of the species or with the use of strong or weak acid. In the HCl-treated series no individual could survive beyond 4 hours at pH 3 and 13 hours at pH 4 after treatment while in the acetic acid series, some of them could survive upto 72 hours or more.

Table 1

Species used	Series	Solution injected	Total sp.	No. of expt.	No. of sp. surv.	Hour of fixation	Mortality %
	Control	0·67% saline	15	3	15	4	Nil
	pH 9	NaOH	30	3	30	4	Nil
	Control	0·67% saline	25	3	25	24	Nil
	pH 9	NaOH	27	3	22	24	19%
Spathosternum prasiniforum	Control	0·67% saline	20	2	20	4	Nil
	pH 3	HCl	20	2	7	4	65%
	Control saline	0·67%	10	2	10	4	Nil
	pH 4	HCl	20	2	8	4	60%
	Control	0·67% saline	20	2	18	18	10%
	pH 4	HCl	20	2	7	18	65%
	Control	Dist. water	5	2	5	24	Nil
	pH 4	Acetic acid	10	2	8	24	20%
Oxya velox	Control	Dist. water	5	2	8	48	Nil
	pH 4	Acetic acid	10	2	5	48	50%
	Control	Dist. water	5	2	5	72	Nil
	pH 4	Acetic acid	10	2	4	72	60%

Further, the higher mortality of grasshoppers due to the injection of the solutions of different pH would be very much apparent when the mortality data is compared with that of control specimens (table 1). Almost cent percent survival of the control specimens indicates that the death of the treated specimens was neither due to the shock of the injection nor due to the fluid used as diluting agents, e.g., 0·67% saline or distilled water.

Testes of each specimen were dissected out and were immediately fixed in

acetic-alchohol mixture. They were squashed and stained either in iron-alum haematoxylin or in Feulgen stain.

Observations

Control Series:

The behaviour of the meiotic chromosomes of the normal individua of the two grasshoppers has been described by Ray Chaudhuri and Dutt (19). The cytological behaviour of the different sets of materials of the control s es resembled those of the normal individuals and showed no abnormality in the chromosomes. In both, the spermatogonial chromosome number has been f d to be 23 and at meiotic prophase there are 12 elements, 11 of them autos d bivalents and the remaining one the unpaired sex chromosome. At metap se I the bivalents undergo much condensation and the univalent sex chrome se lies at any place near the equatorial region. First division anaphase is reducti al.

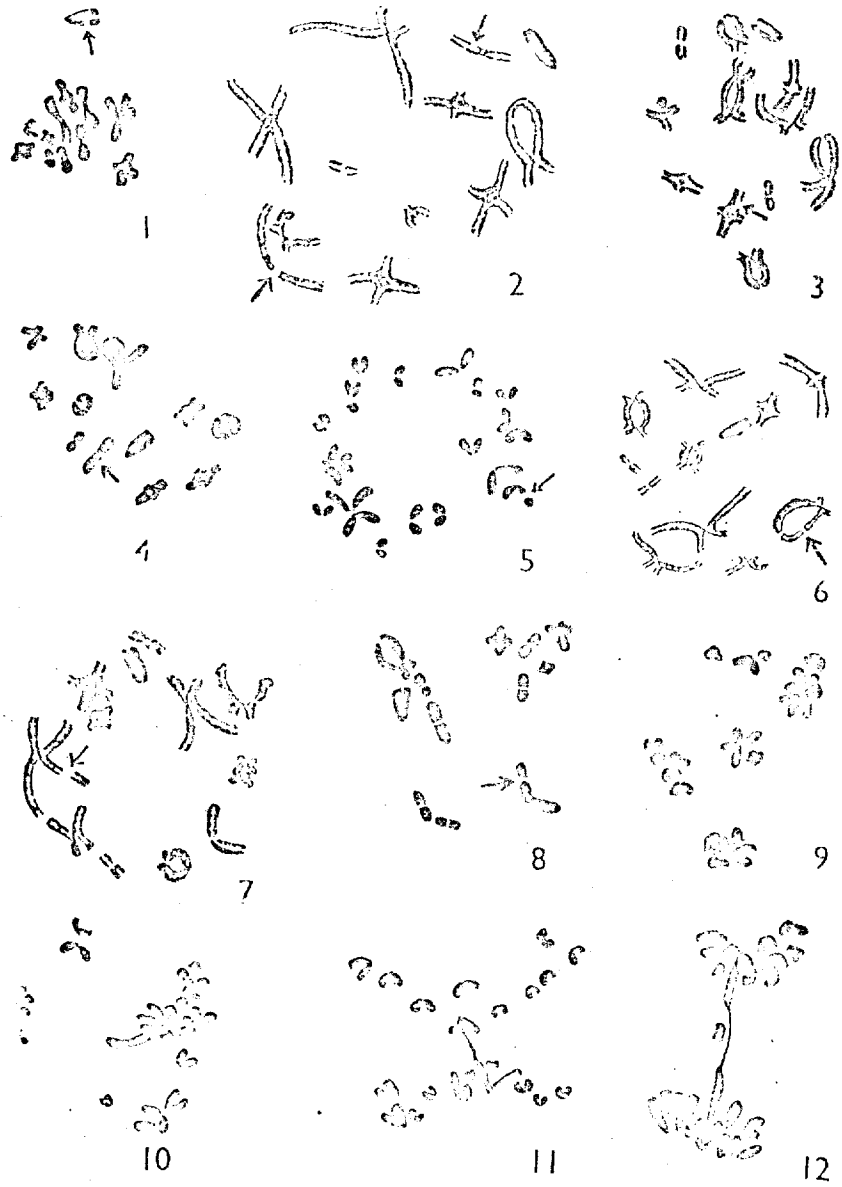
For quantitative study, about one hundred or more meiotic cells of ea h of dividing stage were examined but no cell with chromosomal aberrations like those of treated ones (*vide infra*) was encountered. However, some abnormalities like clumping of chromosomes, sticky anaphase bridge or polyploid cells were encountered in very rare instances.

Treated Series:

Sodium hydroxide solution of pH 9 in Spathosternum prasiniferum: Specimens injected with this solution and testes fixed at 4 hours after injection had very negligible number of cells with chromosome abnormalities. In 100 first spermatocyte late prophase cells not a single cell was found to contain chromosomal aberration of any kind. In 145 metaphase I, only one cell contained a chromatid type break in the X chromosome (fig. 1). Among the limited number of cells available for study, second division metaphase chromosomes were not affected at this hour of fixation. The tissues fixed after an interval of 24 hours of the injection showed marked changes in the spermatocyte chromosomes. Chromatid type breaks were encountered at the different stages of division (figs. 2-5; table 2) while the chromosome type breaks (figs. 6-8) appeared to be more limited in occurrence. First spermatocyte prometaphase stage was found commonly with damages in the spindle structure due to which at metaphase I the bivalents were lying in groups. Since the present study was made from squash preparation.

(Figs. 1-12 are of *S. prasiniferum* treated with sodium hydroxide solution of pH 9. × ca. 1,600).

Fig. 1. Metaphase I with chromatid type break in the X-chromosome. Fig. 2. Diplotene with 2 bivalents showing chromatid and chromosome type break. Fig. 3. Diakinesis with a chromatid type break in a bivalent. Fig. 4. Metaphase I with a chromatid type break in a bivalent. Fig. 5. Polyploid metaphase II with disorganised spindle and a break in a chromosome. Fig. 6. Diplotene with a chromosome type break in a bivalent. Fig. 7. Diakinesis with a chromosome type break in a bivalent. Fig. 8. Metaphase I with disorganised spindle, bivalents arranged in groups, and one of them with a chromosome type break. Fig. 9. Anaphase I with asynchronous division of chromosomes. Fig. 10. Anaphase I with unequal distribution of chromosomes. Fig. 11. Anaphase I with a sticky bridge. Fig. 12. Anaphase I with a bridge and a fragment.


1
2
3
4
5
6
7
8
9
10
11
12

no quantitative data of this type of abnormality was taken as such an abnormality might have also occurred due to the mechanical pressure. However, with the same technique in the control specimens the occurrence was extremely rare.

In the treated specimens the spindle was seen to be affected both at metaphase I and at later stages of division. Various types of abnormalities such as the asynchronous separation of the chromosomes (figs. 9-10), distribution of chromosomes in small groups (fig. 8), extreme stickiness or clumped condition, and sticky bridges (fig. 11) were observed. Some second division metaphase cells were found to be of polyploid constitution which could have originated due to the destruction of the first anaphase spindle (fig. 5). In some cases the chromosome bridge at first division anaphase was formed by structural rearrangement (fig. 12). Second division metaphase and anaphase chromosomes as well as the spindle were also affected with the alterations in pH, resulting in breaks, maldistribution, stickiness and clumping of chromosomes.

Table 2

Frequency of distribution of different types of abnormalities at different stages of meiosis in Spathosternum prasiniferum *injected with NaOH solution of pH 9 and fixed 24 hours after treatment*

	Stage of division and number of cells examined						
	Diplo. (68)	Diak. (74)	Meta. I (246)	Ana. I (182)	Meta. II (176)	Ana. II (70)	Total (816)
Chromatid break	2	2	4	—	4	—	12
Chromosome break	4	1	1	—	—	—	6
Multipolar spindle	—	—	4	6	6	—	16
Polyploid cell	—	1	1	—	2	—	7
Chromosome bridge	—	—	—	4	—	—	4
Lagging and maldistrib.	—	—	—	6	—	4	10
Total	6	4	13	16	12	4	55
Percentage	8·6%	5·4%	5·5%	8·8%	6·8%	5·7%	6·7%

An analysis of the data (table 2) indicates that the percentage of different types of abnormal cells was quite appreciable and all of them contributed to the chromosomal anomalies. The frequency of chromatid and chromosome type breaks was 18 out of 564 cells examined (3·2%).

Hydrochloric acid solution of pH 3-4 in S. prasiniferum: No individual could survive beyond 4 hours after the injection of the solution of pH 3. The effects of

pH 3 and 4 were not qualitatively different. The effect of the acidic pH solutions on the chromosomes as well as on the spindle was similar to those produced by alkaline pH solution. The changes involved were chromosome type and chromatid type breaks, maldistribution of anaphase chromosomes, general stickiness and clumping of the chromosomes, production of polyploid cells, disorganization of the spindle, etc. It appeared that polyploid cells due to fusion of cell boundaries were more prevalent than in other series.

No quantitative data has been scored from the tissues treated with solution of pH 3 since the specimens could not survive for longer period after treatment. Frequency of distribution of abnormalities in the specimens treated with pH 4 has been recorded in table 3.

Table 3

Frequency of distribution of different types of abnormalities at different stages of meiosis in S. prasiniferum *injected with HCl of pH 4 and fixed at 18 hours after treatment*

	Stages of division and the number of cells examined						
	Diplo. (40)	Diak. 83	Meta. I (210)	Ana. I (222)	Meta. II (146)	Ana. II (17)	Total (718)
Chromatid break	1	2	1	—	1	—	5
Chromosome break	—	3	—	—	—	—	3
Multipolar spindle	—	—	—	15	—	—	15
Polyploid cell	—	—	5	5	1	—	11
Chromosome bridges	—	—	—	4	—	—	4
Lagging and maldistrib.	—	—	—	2	—	—	2
Total	1	5	6	26	2	—	40
Percentage	2·5%	6%	2·8%	11·8%	1·4%	—	5·5%

A comparison of the percentage values of the different types of abnormalities (figs 2 and 3) showed that the effect of the solution of pH 9 was relatively more than that of pH 4. However, when the percentages of total abnormal cells were compared the difference between the two was negligible.

Acid solution pH 4 in O. velox: The types of abnormalities observed qualitatively here were more or less like those of *S. prasiniferum* and they did not vary much in the materials fixed at different intervals. The most frequent type of abnormalities was break or gap (figs. 13-15) encountered generally at the late prophase stage of first spermatocyte division. Sometimes instead of having a clear break in a chromosome a constriction was found at particular regions

(fig. 16). Further evidence of breakage was seen from the occurrence of fragmented element in some nuclei (figs. 14, 17). However, the presence of the extra element was not only due to the fragments but also due to the failure of bivalent formation by some chromosomes (fig. 17). A differential despiralization effect in some chromosomes was also encountered (fig. 18). In this effect, sometimes the end of the chromosome appeared to be stretched out and relatively understained. The most common effect observed at the late prophase or metaphase I was the stickiness of the chromosomes. In a number of cases interbivalent connection in the form of thin chromatin threads between the ends of bivalents was observed (fig. 19). The bivalents also were clumped in groups in some cases (fig. 20). Other types of abnormalities were polyploid cells, anaphase bridges, etc. (figs. 21-22).

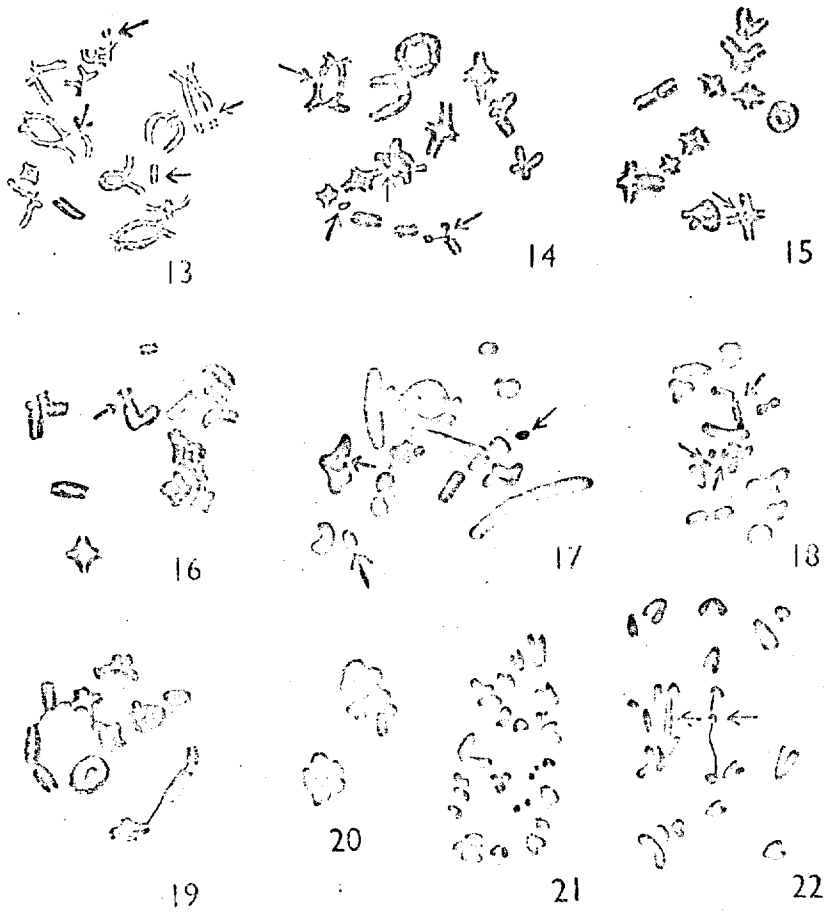
A quantitative study of the frequency of some types of abnormalities in the materials fixed at 24, 48 and 72 hours has been made in table 4.

Table 4

Frequency of distribution of some types of chromosome aberrations in the first meiotic division of Oxya velox *treated with acetic acid solution*

Stage	Hour of fix.	No. of cells	Types of abnormalities				Total	Percentage
			Chroma. break	Chr. break	Constric.	Fragment		
	24	193	2	16	-	3	21	10·8%
Diplotene	48	30	4	1	1	—	6	20·0%
	72	44	—	3	—	—	3	6·8%
	24	414	7	8	2	1	18	4·3%
Diakinesis	48	173	1	1	—	—	2	1·1%
	72	240	3	4	-	1	8	3·3%
Metaphase	24	170	2	3	1	-	6	3·5%
	48	51		—	-	-	0	—
	72	107	1	1	-	1	3	2·7%
Total		1425	20	37	4	6	67	

An analysis of the data roughly indicates that at the diplotene stage the frequency drops down with the enhancement of the fixation period after reaching a peak period at 48 hours. However, this was not evident at diakinesis and metaphase I. The aberration frequency was higher at the diplotene stage. The different types of abnormalities observed show that a good number of cells suffered chromosomal damages as a result of acetic acid solution treatment. Four cells at



g. 13-22 are of *Gyn tera* treated with acetic acid solution of pH 4.5 ca. 1,600 .

13. Diplotene with chromosome type breaks and fragments. Fig. 14. Diakinesis with omosome type breaks, fragments and satellite-like structure. Fig. 15. Diakinesis with a omatid type break. Fig. 16. Diakinesis, a bivalent with a constriction. Fig. 17. Metaphase bivalent with chromatid type break and some univalents and fragments. Fig. 18. Metaphase bivalent with a chromosome type break, another bivalent with despiralised condition and ree extra fragments. Fig. 19. Diakinesis with chain of inter-bivalent connections. Fig. 20. taphase I with extreme clumping effect and bivalents are clumped in groups. Fig. 21. A dyploid metaphase II. Fig. 22. Anaphase I with bridge and fragments.

iakinesis stage and 1 cell of metaphase II (out of 140 cells) were polyploid, cells at anaphase I (out of 144) and 2 cells at anaphase II (out of 144) had chromosome bridges. Therefore, out of a total 1,853 cells 77 or 4·1% cells were with some sort of chromosome aberrations, and thus the total effect (4·1%) was ittle less than that of HCl treatment (5.5%).

This was perhaps due to the fact that the acetic acid treatment did not

produce a significant effect on the spindle structure, and hence cells with multipolar spindle and polyploid cells were rare or absent in comparison with HCl-treatment effects.

Discussion

In the present study although the adult specimens were injected with known strengths of acidic and alkaline pH solutions yet the induced change in the pH of the spermatocyte cells or at the testes itself has not been determined. It is therefore, difficult to state the extent to which the cytological aberrations are due to the change in pH of the cell. Thus, for the sake of accuracy the changes brought about by the pH of the solution should for the present be taken as the changes due to pH shocks until it is established that the injected solutions really changed the pH of the cells to the level of the solution injected.

Since the control specimens injected with only 0·67% saline solution or distilled water and fixed at different intervals of time did not show any cellular changes comparable to those of pH treated ones, possibly the changes observed in the treated series are due to the pH shocks for mixing the chemicals like sodium hydroxide, hydrochloric acid and acetic acid for pH adjustments and not for the injection of the saline solution or the distilled water. The effect of pH shocks upon the spermatocyte cells appears to be quicker since specimens fixed 4 hours after treatment showed qualitatively more or less the same type of chromosomal and spindle abnormalities as those after 18 or 24 hours. However, the frequency of these abnormal types was very low in the materials fixed at 4 hours.

The different kinds of abnormalities observed at different pH may broadly be grouped into two categories namely, (*i*) effects on the spindle and (*ii*) effects on the chromosomes. Qualitatively, the types of effects observed in testes cells due to the pH alterations are similar to those produced by ionizing radiations or radiomimetic chemicals.

If the effect on the spermatocyte cells produced by hydrochloric acid solution with pH adjusted between 3 and 4 (Manna and Ray, 1964) is compared with that of 0·01 M acetic acid solution having the same pH of 3·4, there appears to be some difference with regard to the spindle abnormalities. Hydrochloric acid solution produced relatively more spindle abnormalities while the spindle abnormalities in acetic acid treated cells were negligible. In the former, 19 out of 222 first division anaphase cells were with multipolar spindle, lagging chromosomes, etc. while in the latter such abnormalities were not observed among 144 anaphase cells. Data for some other type of spindle abnormality such as spindle disorganization has not been scored for reasons stated before. But the nature of effects on the chromosome is more or less same between the two types of treatments.

Apparently, different chemical solutions of pH between 3·4 and 9 affect the chromosome of grasshoppers in producing similar kind of effect. Also the response is same even in species belonging to different genera as *Stethophyma grossum* and *Oxya velox* in the present study.

In the quantitative data, the chromosome aberrations could be observed more in the diplotene stage than in any other stage of spermatocyte division. This may either be due to the fact that this stage is most vulnerable or else breaks are produced in equal quantity even at later stages though not detectable due to technical disadvantages. For example, at diakinesis or metaphase the spiralized and over-condensed state of chromosomes makes the detection difficult in many cases. Another reason for obtaining the low frequency could be that the affected cells were fixed before reaching diakinesis or metaphase stage if there was no inhibitory effect. The frequency of abnormal cells at stages later than diplotene was not higher when the fixation hour was extended to 72 hours. Thus it seems more likely that the diplotene stage is relatively vulnerable and perhaps the effect on the chromosome was produced at still earlier stage.

The sodium hydroxide, hydrochloric acid and acetic acid solutions produced chromosome as well as chromatid type breaks. The frequency was found to be higher in most of the cases at the diplotene stage. In the series with acetic acid treatment, the minimum hour of fixation in the present study was 24 hours while in the other two chemicals it was 4 hours after treatment. This indicates that the cells with chromosome type or chromatid type break are likely to have been affected by the chemical at some earlier stage of division. In that case, the occurrence of chromosome and chromatid type breaks may be taken to imply that breaks were produced before and after the chromosome reproduction. Since the chromosome type breaks induced by chemical agents are not likely of the 'isochromatid' type that are produced by ionizing radiations, it is presumed that the bivalent showing the chromosome type break originated before the chromosome reproduction while the chromatid type break in the chromosome was after its reproduction.

The different types of aberration produced by the treatment with acetic acid solution are somewhat comparable to the effect produced by the ionizing radiations or radiomimetic chemicals. However, the effect seems to be more intense in case of ionizing radiations or of some radiomimetic chemicals. With radiations, in most cases, the frequency of aberrations is proportional to the dosage used. However, such results could not be verified because of some obvious limitations. The basic difference between the chromosome aberrations induced by the ionizing radiations and those produced by the chemical agents is that though in both the cases chemically active radicals are produced which indirectly bring about chromosome breakages yet the energy behind the active radicals produced by the ionizing radiations is far greater than those produced by the ionizations of radiomimetic chemicals. Further, on the basis of the dissociation constants of hydrochloric acid and acetic acid the latter is no doubt the weaker acid. As such it was desired to note whether the latter chemical is weaker in action as compared to HCl in inducing chromosome aberrations. In the grand total of HCl treated materials, 5·5%, and in the acetic acid treated material 4·1% cells were abnormal. This comparison should be regarded as a gross one due to various reasons. Apparently there

is no difference in the quality and quantity of the effect between the two kinds of treatments. This is not contrary to expectation, since the two acid solutions are made upto the same pH. The 0·04 M acetic acid solution would dissociate incompletely to give pH 3·4 which was the same for the HCl solution. Although total hydrogen ions are higher in acetic acid, yet the number of active hydrogen ions in both the solutions having the same pH, would be the same. Thus the effect is also expected to be the same. This is in agreement with the present result. Since higher concentration, of either acetic acid or HCl, produce detrimental effect in the tissues, the effect of higher concentrations could not be studied.

Chromosome breaks through chemicals are possibly effected by breaks of hydrogen bond of DNA during the chromosome reduplication. But this assumption is irreconcilliable with the fact that chromatid breaks originate after chromosome reduplication. It may be suggested that the change in the ion activity around the nucleus would lead to the exchange of ions between the nucleus and its surrounding which may result in various chromosomal and spindle abnormalities as manifested in these experiments. It implies that the action of acetic acid is different from the effect of HCl solution. Though both may produce similar type of chromosome aberrations, the former is less effective in causing damage to the spindle structure. The spindle structure is known to be mainly formed of fibrous protein. At present it is not known if the fibrous protein of the spindle is comparable to that present in chromosome structure.

Summary

Adult male grasshoppers, *Spathosternum prasiniforum*, were injected abdominally with 0·02 ml. NaOH solution of pH 9 and HCl solutions of pH 3 and 4 and fixed at intervals of 4, 18, 24 hours. Specimens injected with 0·75% saline solution served as the control. Similarly specimens of another species, *Oxya velox*, were injected with 0·04 M acetic acid solution of pH 3·4 and fixed at 24, 48, 72 hours. Specimens injected with distilled water served as the control.

Survival percentage was low in the specimens treated with solutions of different pH and it was still lower in hydrochloric acid solutions. Specimens treated with acetic acid solution could survive longer than those treated with sodium hydroxide or hydrochloric acid solution.

The nature of damages caused in the spermatocyte cells due to the treatment with solutions of different pH have been studied both qualitatively and quantitatively. All types of treatments produced chromatid and chromosome type breaks, fragments, constrictions, chromosome bridges at anaphase, interbivalent connections, polyploid cells, multipolar spindle, etc. The frequency data for some of the abnormalities have been scored and no appreciable difference in the frequency was observed between the different types of treatment. The frequency of abnormalities associated with spindle structure was found to be less in acetic acid-treated materials. Diplotene stage was found to be more susceptible to damages in the chromosomes.

The probable mechanism in the production of the various kinds of abnormalities by the solutions of different pH has been discussed. Since in the control specimens meiotic abnormalities similar to those in treated ones were absent, it has been assumed that the changes were caused due to the quantitative alterations of the active ions.

REFERENCES

AUERBACH, C. 1951. Some recent results with chemical mutagens. *Hereditas*, **37**: 1-16.

—— & ROBSON, J. M. 1947. Tests of chemical substances for mutagenic action. *Proc. Roy. Soc. Edinb.* B, **62**: 271-291.

AULA, P. 1955. Virus-associated chromosome breakage. A cytogenetic study of chicken-pox, measles, and mumps patients and of cell cultures infected with measles virus. *Ann. Acad. Sci. Fenn.*, Sr. A. Sr. IV, No. **36**: 1-73.

BACQ, A. M. & ALEXANDER, P. 1955. Fundamentals of radiobiology. Academic Press, New York.

BIESLE, J. J. 1958. Mitotic poison and the cancer problem. Elsevier Press, New York.

D'AMATO, F. 1959. The chromosome breaking activity of chemicals as studied by Allium cepa test. *Publ. Stat. Zool. Napoli*, **22** (Suppl.): 158-170.

EIGSTI, O. J. & DUSTIN, P. 1957. Colchicine. In Agriculture, Medicine, Biology and Chemistry. Iowa State College Press, Iowa, U.S.A.

GRUNDMANN, E. 1964. Allgemeine cytologie. Stuttgart.

HOLLAENDER, A. 1954. Radiation biology. Vol. 1. McGraw-Hill Book Company, Inc., New York.

HSU, T. C. & SOWERS, C. E. 1961. Effect of 5-bromodeoxyuridine on mammalian chromosomes. *Proc. Nat. Acad. Sci.* (Wash.), **47**: 396-403.

KHILMAN, B. A. 1956. Factors affecting the production of chromosome aberrations by chemicals. *J. Biophys. Biochem. Cytol.*, **2**: 543-555.

——. 1961. Biochemical aspects of chromosome breakage. *Adv. Genet.* **10**: 1-59.

MANNA, G. K. & MAZUMDER, S. C. 1964. Ethyl alcohol induced sex chromosome breakage in the grasshopper, *Phlocoba antennata*. *Naturwissen.*, **24**: 646.

—— & PARIDA, B. B. 1965a. Differentially administered colchicine effect upon the testis cells of the grasshopper, *Spathosternum prasiniferum*. *Cytologia*, **30**: 392-401.

—— & ——. 1965b. Aluminium chloride induced meiotic chromosome aberrations in the grasshopper, *Phlocoba antennata*. *Naturwissen.*, **23**: 647-648.

—— & ——. 1966. Formalin induced sex chromosome breakage in spermatocyte cells of the grasshopper *Traloria palmata* Uvarov. *Proc. 53rd Ind. Sci. Cong.* Pt. **III** (Abstract): 321.

—— & RAY, D. 1964. Grasshopper spermatocyte chromosomal changes induced by pH alterations. *Naturwissen.*, **23**: 493-494.

MEHLKERS, F. 1913. Die-Auslosung von chromosomen mutationen in der meiosis druch Einwirkung von Chinin-salzen. *Zeitscher. ind. Abstam. Vererbungsl.*, **81**: 313-314.

RAY-CHAUDHURI, S. P. 1961. Induction of chromosome aberrations in the spermatocytes of grasshoppers. *Nucleus*, **4**: 47-66.

—— & DUTT, M. K. 1947. A comparative study of the chromosome structure and behaviour in three genera of Indian grasshoppers. *Proc. Roy. Soc. Edinb.*, **62**: 292-298.

SARKAR, J. 1959. Effect of nitrogen mustard on two species of grasshoppers. *Proc. Zool. Soc.* (Cal.), **9**: 115-121.

SHARMA, A. K. & SHARMA, A. 1960. Spontaneous and chemically induced chromosome breaks. *Int. Rev. Cytol.*, **10**: 101-136.

WILSON, G. B. 1960. The study of drug effects at the cytological level. *Int. Rev. Cytol.*, **9**: 293-304.

YOSHIA, T. 1959. Some observations on abnormal nuclear division in grasshopper testes treated with colchicine *Jour. Fac. Sci. Hokkaido Univ.* Sr. 6, **10**: 61-70.

Biosynthesis of Cholesterol and Fatty Acids from Acetate - 2- ^{14}C in Various Organs and Tissues of Rats

V. F. Markelova and V. A. Tatsievskii (Institute of Nutrition of the USSR Academy of Medical Sciences, Moscow).

Source: Voprosy Meditsinskoi Khimii 12(6) : 646-7, 1966.

It is known that the liver constitutes the principal point (location) of cholesterol biosynthesis. In smaller amounts, cholesterol is synthesized in the adrenal cortex, the intestinal wall, the skin and in insignificant amounts in other organs and tissues (1, 2, 3). It is also known that in rabbits, guinea pigs and chicks the aorta is capable of synthesizing cholesterol whereas this process does not take place in the aorta of man, dogs, cats, and rats (4, 5).

This article gives the results of a study of the biosynthesis of cholesterol and fatty acids from acetate-2-^{14}C in various rat tissues.

The study was carried out on 10 male Wistar rats, weighing about 300 g, which were kept on a briquet food diet. The acetate-2-^{14}C was introduced subcutaneously into the inner surface of the left hip in a volume of 1 ml, calculated for about 35,000 impulses per gram body weight. Two hours after injection of the labeled acetate, the animals were killed. To record the total radioactivity entering into the organs, counting was done in a thick layer of crude tissue preparations with the aid of an end-window counter BFL-T-25 in a PP-8 unit. Cholesterol was isolated by the modified Sperry-Webb method; 250 mg of tissue was hydrolyzed in a 15% alcoholic KOH solution for 3 hours on a water bath equipped with a reflux condenser. The hydrolyzed product was extracted with petroleum ether (b.p. 40-60°C). The extracts

QUESTIONNAIRE REVIEW FORM ___ ___ - ___ ___ ___ ___ - ___ ___ ___

The following items should be reviewed.

Page	Item	Code	

Definition of Codes:

1. Omission of relevant data.
2. Not within guidelines.
3. More than one response.
4. Requires regional approval.
5. Other problems

7/03/73

were washed with 10% KOH, distilled water, 10% acetic acid and again with distilled water, dried with Na_2So_4, filtered through a degreased paper filter, and evaporated to dryness on a water bath; 5 ml of a 1:1 alcohol-acetone mixture and 2 ml of a 0.5% digitonin solution in 80% ethyl alcohol were then added. After 24 hours, the cholesterol digetonide deposit was precipitated by centrifuging at 5000 rpm and transferred to previously weighed targets. After the targets were dried, they were again weighed and subjected to counting in the radiometric unit. For the isolation of fatty acids, the aqueous-alkaline phase remaining after extraction of cholesterol was acidified with 5N H_2So_4 to a weakly acid reaction, using methyl orange as indicator. The fatty acids were extracted with petroleum ether, the extract was washed with distilled water, dehydrated with Na_2So_4 and evaporated to dryness on the water bath. The dry fatty acid residue was dissolved in a small amount of ether and transferred to the targets.

The radioactivity of cholesterol and fatty acids was expressed in units of specific activity, relative specific activity and in percent of acetate-2-^{14}C incorporation.

As can be seen from the data listed in the table, all organs and tissues tested show incorporation of labeled acetate into cholesterol. The highest specific radioactivity was found in cholesterol of blood, liver, lungs, kidneys and spleen. The relative specifie radioactivity was highest in adrenal glands, liver, spleen, lungs and kidneys. The percentage of acetate-2-^{14}C utilization in the synthesis of cholesterol was highest in adrenal glands, liver, lungs, spleen and kidneys. The above data show that the total amount of cholesterol synthesized in the lungs and spleen is, apparently, about equal to the amount of cholesterol synthesized in the liver.

Table: Incorporation of acetate-2-^{14}C into cholesterol of rat organs and tissues.

1 Орган и ткань	2 Удельная радиоактивность холестерина	3 Относительная удельная радиоактивность холестерина (в %)	4 включение 2-C^{14}-ацетата в холестерин
5 Кровь . . .	33,0±5,20	0,19±0,01	0,41±0,04
6 Сердце . . .	9,5±1,55	0,11±0,02	0,36±0,01
7 Легкие . . .	20,0±1,44	0,68±0,08	0,86±0,02
8 Печень . . .	29,0±2,17	1,01±0,07	1,49±0,13
9 Селезенка	19,0±0,91	0,71±0,01	0,71±0,06
10 Почки . . .	20,6±0,67	0,68±0,05	0,71±0,04
11 Мозг . . .	3,0±0,01	0,26±0,02	0,47±0,01
12 Аорта . . .	8,0±0,01	0,08±0,001	0,09±0,001
13 Надпочечники . . .	11,5±1,50	1,93±0,87	2,01±0,1

1 - Organ and tissue
2 - Specific activity of cholesterol
3 - Relative specific activity of cholesterol (in%)
4 - Incorporation of acetate-2-^{14}C into cholesterol (in%)
5 - Blood
6 - Heart
7 - Lungs
8 - Liver
9 - Spleen
10 - Kidneys
11 - Brain
12 - Aorta
13 - Adrenal glands

The relative specific radioactivity of fatty acids in the liver was 1.32, in the blood 0.11, in the heart 0.60, and in the spleen 1.65. The percentage utilization of acetate-2-^{14}C for the synthesis of fatty acids in the liver was 1.32, in the blood 0.41, in the heart 1.21, and in the spleen 1.35.

The data obtained indicate that, in studying cholesterol metalolism, we must take into account not only the liver, but also other organs and

QUESTIONNAIRE REVIEW FORM ___ ___ - ___ ___ ___ ___ - ___ ___ ___

The following items should be reviewed.

Page	Item	Code	

Definition of Codes:

1. Omission of relevant data.
2. Not within guidelines.
3. More than one response.
4. Requires regional approval.
5. Other problems

7/03/73

primarily the lungs and spleen, the relative importance of which in cholesterol metabolism is not as small as is generally assumed at the present time.

Bibliography

1. Kritsman M. G., Babina M. V., Biokhimiya 1955, no. 5, p. 585.
2. Blagorazumova M. A. Inbook: Sbornik nauchn. rabot Volgogradsk. med. in-ta, Volgograd, 1957, vol. 11, p. 28.
3. Tret'yakova K. A., Grodzenskii D. E., Vopr. med. khimii 1959, no.5, p. 362.
4. Azarnoll D. E., Curran G. L., Williamson H. P., Ted Proc., 1957, vol. 16, p. 148.
5. Werthessen N. T., Nvman M. A., Holman R. L. et al, Circulat. Res, 1956, vol. 4, p. 586.

Received for publication
on 20 April 1966

QUESTIONNAIRE REVIEW FORM ___ ___ - ___ ___ ___ ___ - ___ ___ ___

The following items should be reviewed.

Page	Item	Code	

Definition of Codes:

1. Omission of relevant data.
2. Not within guidelines.
3. More than one response.
4. Requires regional approval.
5. Other problems

7/03/73

БИОСИНТЕЗ ХОЛЕСТЕРИНА И ЖИРНЫХ КИСЛОТ ИЗ 2-АЦЕТАТА В РАЗЛИЧНЫХ ОРГАНАХ И ТКАНЯХ КРЫС

В. Ф. Маркелова, В. А. Тацневский

Институт питания АМН СССР, Москва

Vop. Med. Khim. 12(6): 646-7, (1966)

Известно, что основным местом биосинтеза холестерина является печень. В меньшем количестве он синтезируется в коре надпочечников, стенке кишечника, коже и в незначительных количествах — в других органах и тканях [1, 2, 3]. Известно также, что у кроликов, морских свинок и цыплят аорта обладает способностью синтезировать холестерин, в аорте же человека, собаки, кошки и крысы указанный процесс не имеет места [4, 5].

В настоящем сообщении приводятся результаты изучения биосинтеза холестерина и жирных кислот из 2-C^{14}-ацетата в различных тканях крысы.

Исследования проводили на 10 крысах-самцах породы Вистар, весом около 300 г, вскармливавшихся брикетированным кормом. 2-C^{14}-ацетат вводили подкожно в область внутренней поверхности левого бедра в объеме 1 мл из расчета ≈25 10^{6} импульсов на 1 г веса. Через 2 часа после введения препарата животных забивали. Для регистрации общей радиоактивности, поступающей в органы, препараты сырых тканей просушивали в толстом слое с помощью торцового счетчика БФЛ 25 на установке ПП-8. Холестерин выделяли по модифицированному способу Сперри — Вебба. 250 мг ткани гидролизовали в 15% спиртовом растворе КОН в течение 3 часов на водяной бане с обратным холодильником. Гидролизаты экстрагировали в делительной воронке эфиром (т. кип. 40—60°). Экстракты промывали 10% КОН, дистиллированной водой, 10% уксусной кислотой и вновь дистиллированной водой, высушивали Na_2SO_4, фильтровали через обезжиренные бумажные фильтры, упаривали досуха на водяной бане, добавляли 5 мл смеси спирт — ацетон (1 : 1) и 2 мл 0,5% раствора digitonina в 80% этиловом спирте. Через 24 часа осадок холестеридигитонина осаждали центрифугированием при 5000 об/мин и переносили на предварительно взвешенные миллиметровые диски. После подсушивания дисков вновь взвешивали и определяли на радиометрической установке. Для выделения жирных кислот водно-щелочную фазу, оставшуюся после экстракции холестерина, подкисляли 5 н. H_2SO_4 до слабокислой реакции по метил-красному. Жирные кислоты экстрагировали петролейным эфиром. Экстракт промывали дистиллированной водой, обезвоживали Na_2SO_4 и упаривали досуха на водяной бане. Сухой остаток жирных кислот растворяли в небольшом количестве эфира и переносили на диски.

Радиоактивность холестерина и жирных кислот выражали в единицах удельной радиоактивности, относительной удельной радиоактивности и в процентах включения 2-C^{14}-ацетата.

Как видно из данных, представленных в таблице, все исследованные органы и ткани включают меченый ацетат в холестерин. Наибольшей удельной радиоактивностью обладал холестерин крови, печени, легких, почек, селезенки. Относительная удельная радиоактивность была самой высокой в надпочечниках, печени, селезенке, легких и почках. Процент использования 2-C^{14}-ацетата для синтеза холестерина был самым высоким в надпочечниках, печени, легких, селезенке, почках. Из описанных выше данных следует, что количество холестерина, синтезируемого в легких и селезенке, суммарно, по-видимому, приблизительно равно количеству холестерина, синтезируемого в печени.

Относительная удельная радиоактивность жирных кислот печени была равна 1,32, крови — 0,11, сердца — 0,60, селезенки — 1,65. Процент использования 2-C^{14}-ацетата для синтеза жирных кислот в печени составлял 1,32, в крови — 0,41, сердце — 1,21, селезенке — 1,35.

Полученные данные свидетельствуют о том, что при изучении метаболизма холестерина следует учитывать не только печень, но и другие органы, в первую очередь легкие и селезенку, удельный вес которых в обмене холестерина не так уж мал, как это принято считать в настоящее время.

Включение 2-C^{14}-ацетата в холестерин органов и тканей крыс

Орган и ткань	Удельная радиоактивность холестерина	Относительная удельная радиоактивность холестерина (в %)	Включения 2-C^{14}-ацетата в холестерин
Кровь . . .	33,0±5,20	0,19±0,01	0,41±0,04
Сердце . . .	9,5±1,55	0,11±0,02	0,36±0,01
Легкие . . .	20,0±1,44	0,68±0,08	0,86±0,02
Печень . . .	29,0±2,17	1,01±0,07	1,49±0,13
Селезенка	19,0±0,91	0,71±0,04	0,71±0,06
Почки . . .	20,6±0,67	0,68±0,05	0,71±0,04
Мозг . . .	3,0±0,01	0,26±0,02	0,47±0,01
Аорта . . .	8,0±0,01	0,08±0,001	0,09±0,001
Надпочечники . . .	11,5±1,50	1,93±0,87	2,01±0,1

ЛИТЕРАТУРА

1. Крицман М. Г., Бавина М. В. Биохимия, 1955, в. 5, с. 585. — 2. Благоразумова М. А. В кн.: Сборник научн. работ Волгоградск. мед. ин-та. Волгоград, 1957, т. 11, с. 28. — 3. Третьякова К. А., Гродзенский Д. Э. Вопр. мед. химии, 1959, в. 5, с. 362. — 4. Azarnoff D. E., Curran G. L., Williamson H. P., Fed. Proc., 1957, v. 16, p. 148. — 5. Werthessen N. T., Nyman M. A., Holman R. L. et al., Circulat. Res., 1956, v. 4, p. 586.

Поступила 20.IV 1966 г.

Вопросы медицинской химии, 1966 г., т. XII, в. 6

EUROPEAN JOURNAL OF PHARMACOLOGY 11 (1970) 38–47. NORTH-HOLLAND PUBLISHING COMPANY

TOXICITY OF ACIDS AND BASES AFTER INTRAPERITONEAL INJECTION *

Lawrence MARTIN ** and Aaron H. ANTON ***
Departments of Pharmacology and Anesthesiology,
University of Florida College of Medicine,
Gainesville, Florida 32601, USA

Received 24 September 1969 Accepted 3 April 1970

L.MARTIN and A.H.ANTON, *Toxicity of acids and bases after intraperitoneal injection* European J. Pharmacol. 11 (1970) 38–47.

Acetic acid, injected intraperitoneally, was lethal for mice housed in an incubator (37°C) and in the cold room (5°C), but not at room temperature (23°C). A dose of 4 meq/kg killed over 50% of the mice within 4 hr at the extremes of temperature, and none at 23°C. This difference in mortality was also obtained with other irritating acids and bases at the same dose, but not their salts. This effect was dose dependent with respect to the milliequivalency of injected agent.

The cause of death is unknown. Poikilothermia was induced with the lethal agents at all 3 temperatures. Pretreatment of the mice with various pharmacologic agents did not prevent death. Autopsy of animals sacrificed just prior to death revealed necrosis of the mucosa and a peritoneal exudate in acid-treated animals; this pathology was judged identical at 23°C and 37°C with the same dose of acid.

This pattern of mortality and poikilothermia resembles that obtained with endotoxin in mice, and may provide a simple, reproducible model for studying "endotoxic" death in mice, as well as its possible pharmacologic prevention.

Shock model
Drug-induced poikilothermia
Temperature and drug interaction
Acid toxicity
Base toxicity

1. INTRODUCTION

During studies on the interaction between an elevated ambient temperature and certain drugs on catecholamine metabolism in mice, we found that acetic acid, which was used to solubilize the drugs, was highly toxic. The lethality of the acid was dose and temperature related, and was common to certain acids and bases when injected intraperitoneally. This report describes our attempts to elucidate the nature of this phenomenon which resembles the effects as described in the literature after the administration of endotoxins.

* Supported, in part, by National Heart Institute Grant HE-05251.

** This project was carried out by this author during the Experimental Medicine course at this institution.

*** Professor of Anesthesiology and Pharmacology. Address for reprints: A.H. Anton, Ph.D., Department of Anesthesiology, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106, USA.

2. METHODS

We used 25–30 g 2–3 months old male mice strain ICR, from Dublin Laboratories, Dublin, Virginia. The mice were housed 10 per cage for a minimum of 24 hr before use, and food and water were supplied *ad libitum* prior to and during all experiments.

The desired dose of each drug, freshly prepared, was injected intraperitoneally (i.p.) in a constant volume of 0.1 ml, unless otherwise indicated. The three temperatures used in these experiments were 37°C (incubator), 23°C (room temperature) and 5°C

cold room). Most of the experiments were done with 10 mice per cage.

Body temperature was measured rectally with a Tele-Thermometer (Yellow Springs Instrument Co., Model 46TUO). In a few experiments we determined the norepinephrine content of brain and heart (Anton and Sayre, 1962).

All experiments were performed approximately the same time in the afternoon to minimize circadian variation.

3. RESULTS

During a preliminary study on the effect of temperature on catecholamine metabolism, 2 groups of mice were each injected i.p. with reserpine, tetrabenazine, syrosingopine, or guanethidine. Then one group was placed in the incubator and the other group left at room temperature. Unexpectedly, the incubator-housed animals given reserpine, tetrabenazine and syrosingopine were found dead after 2 hr, whereas the mice receiving guanethidine were alive; also, all those kept at room temperature were alive.

The central amine-depleting effect of reserpine and tetrabenazine (Shore, 1962) and the associated depression was not the cause of death since there was no correlation between depletion of brain norepinephrine and toxicity; thus, syrosingopine, which did not deplete the brain, was equally toxic to reserpine and tetrabenazine, which did deplete. (Brain nor-

Table 1
The interaction between temperature and drugs on mouse survival: an indication of the solvent effect.

Drug [a]	Dose (mg/kg)	Solvent [b]	Percent dead [c]			
			37°C			23°C
			1 hr	2 hr	4 hr [d]	4 hr
Experiment A						
Buffered saline, pH 7.0			0	0	0	0
Reserpine	5	C	0	60	100	10
Reserpine	3	C	0	60	90	0
Guanethidine	10	S	0	0	0	0
Tetrabenazine	50	C	0	90	100	0
Syrosingopine	4	C	0	40	80	0
Phenobarbital	80	S	0	0	0	0
Chlorpromazine	10	S	0	0	0	0
Lyophilized soluble reserpine phosphate	4	S	0	0	0	0
Combination solvent without drug		C	10	30	50	0
Experiment B						
Buffered saline, pH 7.0			0	0	0	0
Acetic acid (4 meq/kg)			0	20	50	0
Ethanol, 7%			0	0	0	0
Propylene glycol, 15%			0	0	0	0
Acetic acid (4 meq/kg) + guanethidine (10 mg/kg) [e]			0	60	80	0

[a] Injected as 0.1 ml total solution, intraperitoneally.
[b] C = Combination solvent; consisting of 5% acetic acid, 7% ethanol, 15% propylene glycol, and water.
S = Saline solvent; this was 0.15 M with respect to NaCl and 0.01 M with respect to phosphate at pH 7.0.
[c] Based on 10 mice for each drug at each temperature.
[d] In this and subsequent experiments the mortality with acetic acid was maximal by 4 hr; it was rare for a mouse to die after the fifth hour.
[e] The acetic acid and guanethidine were mixed prior to the injection.

epinephrine data are not shown since no correlation with toxicity was evident). Chlorpromazine and phenobarbital markedly depressed the animals, but did not kill them; these agents did not lower brain norepinephrine levels.

The toxicity was found to be due to the solvent for these amine-depleting bases, and not the drug *per se* (table 1, Exp. A). Reserpine, tetrabenazine and syrosingopine bases had been solubilized with acetic acid, whereas guanethidine, chlorpromazine and phenobarbital were used as water-soluble salts. Also, a lyophilized reserpine preparation, which depleted central and peripheral stores of catecholamines, was not toxic when saline was used as the diluent (table 1, Exp. A). Phenobarbital and chlorpromazine were used to rule out a nonspecific depressant effect as the toxic component.

Although propylene glycol and ethanol also were part of the solvent system for the bases, the toxicity was found to be due specifically to the acetic acid * (table 1, Exp. B). Interestingly, the mortality data in table 1 suggest that the combination of an amine depletor (Shore, 1962; Orlans et al., 1960) whether central (tetrabenazine), peripheral (syrosingopine and guanethidine) or both (reserpine), plus acetic acid, is at 37°C more toxic than the acid alone.

* The mice had been receiving about 4 meq/kg of acetic acid which happened to approximate the LD_{50}. Therefore this dose was used in these experiments, and it was obtained by injecting 0.1 ml of a 1 N solution.

3.1. *Dose relationship*

That the effect is dose-related is shown in table 2: one meq/kg of acetic acid had no effect at either temperature, whereas 12 meq/kg was toxic at both temperatures. Also at 37°C the higher amount of acid was more toxic than the 4 meq/kg dose since it killed all the mice, and the mortality appeared earlier.

Administration of HCl and NaOH gave the same picture as acetic acid at both temperatures, and NaOH seemed to be the most toxic of the three. The acidic and basic salts of these agents (NH_4Cl, sodium acetate) gave no deaths at either temperature.

A pH of 0.1 with HCl produced no greater death rate at 4 hr than a pH of 2.4 with acetic acid or 3.25 with acetic acid in buffered saline (table 2). Though the highest dose of acetic acid had three times the chemical equivalency than did the HCl, its H^+ concentration was only 1/100 as great; and since the death

Table 2
The effect of various agents on survival of mice at 37° and 23°C.

Agent [a]	Normality	meq/kg	Solution pH	Percent dead [b] 37°C 1 hr	37°C 2 hr	37°C 4 hr	23°C 4 hr
Buffered saline	0.16		7.00	0	0	0	0
Acetic acid	0.25	1	2.65	0	0	0	0
	1.0	4	2.40	0	20	50	0
	3.0	12	2.10	30	90	100	60
	1.0	4	3.25 [c]	0	30	70	0
HCl	1.0	4	0.10	0	0	60	0
NaOH	1.0	4	12.40	10	70	90	0
Salts							
Sodium acetate	1.0	4	8.80	0	0	0	0
Sodium bicarbonate	1.0	4	7.80	0	0	0	0
Ammonium chloride	1.0	4	4.85	0	0	0	0

[a] Injected as 0.1 ml total solution, intraperitoneally.
[b] Based on 10 mice for each agent at each temperature.
[c] In buffered saline, pH 7.0.

te with the 12 meq/kg dose of acetic acid was gher, the toxicity was due to the meq of acid jected, and not to the pH *per se*.

2. Body temperature

A) *Exposure to 23°C and 37°C.* Because these gents might be interfering with thermoregulation, ectal temperatures were measured in mice given both olvents as well as the various amine depletors, and xposed to 23 and 37°C (figs. 1 and 2). The data in hese graphs indicate that at 23°C those solutions ontaining the acid tended to make the animals oikilothermic. In the incubator, the animals appeared to show a spike in temperature at 2 hr and a decline at 4 hr toward normal body temperature. But of the 5 mice given tetrabenazine, only 1 lived an hour, and none were alive at 2 hr. Of the 5 test animals treated with syrosingopine, 3 lived 2 hr, and only 1 lived 4 hr. Thus, in the case of those mice receiving acetic acid, only a few of those whose temperature was taken at zero time survived to have their temperature taken again at the end of the experiment. This apparent interference with thermoregulation appeared to be enhanced by the addition of an amine depletor (figs. 1 and 2), a finding consistent with the increased mortality from the combination. Note that the temperature imbalance occurred in the first 10–15 min after the animal received the injection of the acid-containing solution.

B) *Exposure to 5°C.* Exposing the mice to a cold environment provided further evidence for an acid-induced poikilothermia. In this experiment the rectal temperatures of the mice were taken at 23°C just prior to the intraperitoneal injection of acetic acid or saline (no other drugs were used). The mice, housed either singly or in groups of 10, were then immediately placed in the cold room (5°C). As shown in fig. 3 there was a marked decrease in rectal temperature of the acid-treated animals and the effect was significantly greater in the individually-housed mice. All of the acid-treated, individually-housed animals were dead after 4½ hr in the cold room whereas all of the others survived. At 37°C we had not found group size to be a factor in mortality, having obtained similar death rates when mice were grouped 1, 5, 10 or 15 per cage.

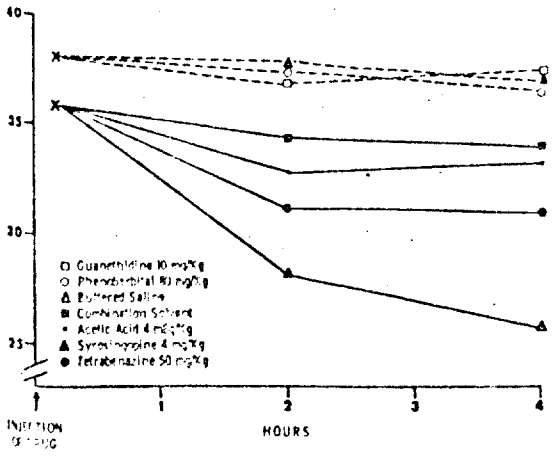


Fig. 1. The effect of various drugs on rectal temperatures of mice at 23°C ambient. Each symbol represents an average of temperatures from 5 different mice over the 4-hr period. Guanethidine and phenobarbital were dissolved in saline (open symbols); syrosingopine and tetrabenazine were dissolved in the combination solvent containing acetic acid (closed symbols). Note the approximately 2°C decrease in initial body temperature within 10–15 min in the acid-treated animals.

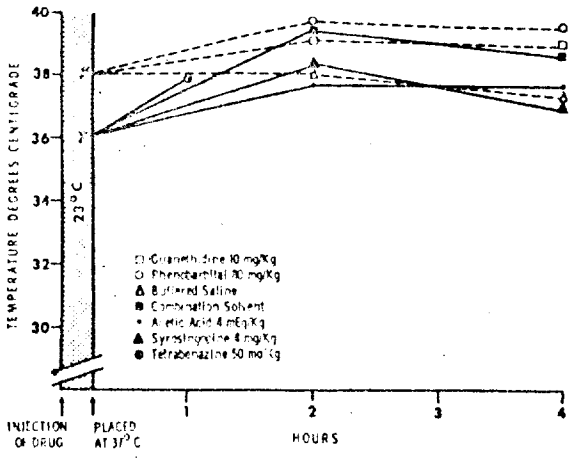


Fig. 2. The effect of various drugs on rectal temperatures of mice at 37°C ambient. Temperatures were initially determined at 23°C within 10–15 min after injecting the drug. Note the 2°C decrease in body temperature within 10–15 min in the acid-treated animals (closed symbols). In this figure only the initial temperatures are the averages from the 5 mice in each group. In no case did all the acid-treated animals live 4 hr. In the case of tetrabenazine all the mice were dead by 2 hr.

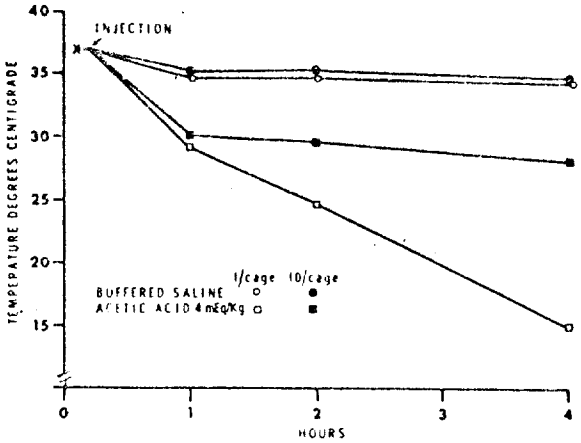


Fig. 3. The effect of acetic acid on rectal temperatures of mice at 5°C ambient. Each symbol represents the average of temperatures from 5 different mice over the 4-hr period. Initially, the temperatures were taken at 23°C just prior to the intraperitoneal injection of the acetic acid or saline. The mice, housed singly or in groups of 10, were then immediately placed in the cold room at 5°C. Temperatures were significantly different at the two tailed 5% level (or less) between those groups whose respective symbols are shown to be some distance apart.

3.3. *Respiratory effect*

The possibility that some of the animals at 37°C might be dying of respiratory depression seemed unlikely since the anesthetic dose of an aqueous solution of barbiturate, which depressed respiration, was not lethal at 37°C (table 1). It is conceivable, however, that under these conditions the acid depressed respiration more than the barbiturate.

3.4. *Pharmacologic intervention*

Numerous drugs of a diverse pharmacologic nature were tried in an attempt to gain some insight into the mechanism responsible for the lethal action of the acid (table 3). These agents were selected because of their use in studies on the treatment of various forms of shock in animals (Lillehei et al., 1964; Zweibach, 1963). None afforded protection except prednisolone (20 and 40 mg/kg) given 5 min before the acid. A longer interval of pre-treatment with the steroid seemed to afford no protection. Post-treatment with steroids also afforded no protection (not shown in table 3). Pre-treatment with intraperitoneal glucose gave marked protection, whereas subcutaneous glucose gave none.

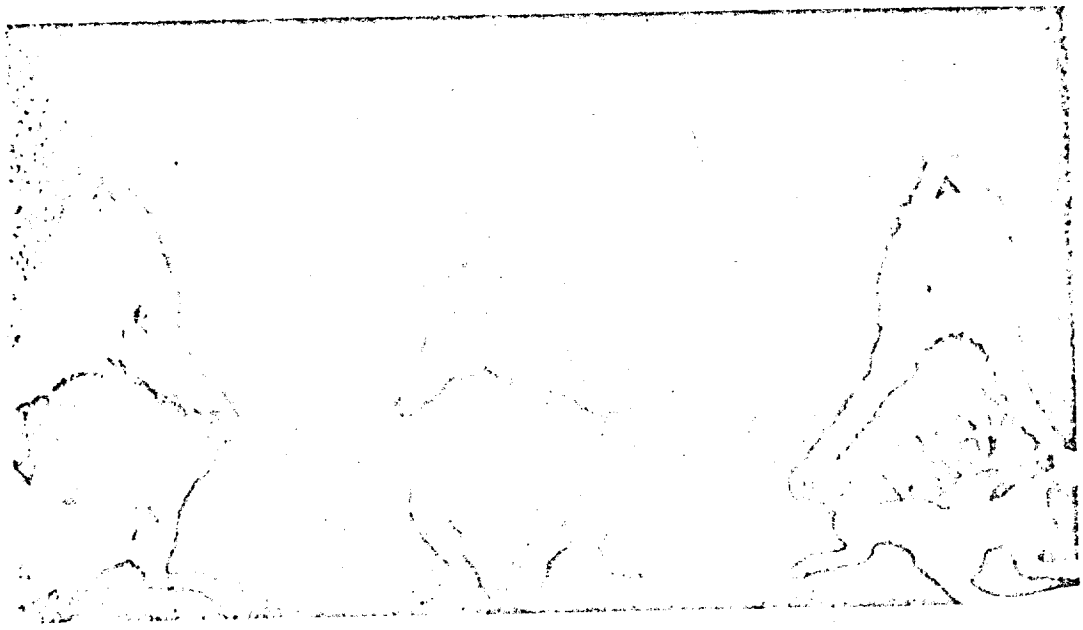
Table 3
Pharmacologic attempts to prevent acid toxicity at 37°C

Treatment [a]	min before acid [b]	number of mice [c]	% dead by 4 hr
Control		200	67.5
Prednisolone (20 mg/kg)	5	30	37
Prednisolone (20 mg/kg)	180	10	60
Prednisolone (40 mg/kg)	5	10	30
Prednisolone (60 mg/kg)	15	20	70
Salt medium for prednisolone [e]	5	10	90
Normal saline (1.0 ml) [d]	5	40	65
Normal saline (2.0 ml) [d]	5	10	60
25% glucose (1.0 ml)	15	30	15
25% glucose (1.0 ml) [d]	15	20	90
8.5% NaCl (1.0 ml)	15	10	70
Chlorpromazine (10 mg/kg)	5	10	80
Chlorpromazine (10 mg/kg) [d]	5	10	90
Chlorpromazine (10 mg/kg) [d]	30	10	100
Propranolol (10 mg/kg)	10	10	80
Phenoxybenzamine (10 mg/kg)	30	10	90
Prednisolone (20 mg/kg) + phenoxybenzamine (2 mg/kg)	15	10	60
Penicillin G (20 × 10⁶ units/kg)	30	20	60
Penicillin G (48 × 10⁶ units/kg)	30	10	70
Chloramphenicol (2 g/kg)	30	10	80
Penicillin G (20 × 10⁶ units/kg) + chloramphenicol (1–2 g/kg)	30	10	50
Morphine (10 mg/kg)	15	10	70
Pentazocine (10 mg/kg)	15	10	70
Levoprome (10 mg/kg)	15	10	80
Lidocaine (10 mg/kg)	5	10	80
Lidocaine (25 mg/kg)	5	10	70
Procaine (25 mg/kg)	5	10	90

[a] All drugs injected in a total volume of 0.1 ml except where indicated.
[b] After the i.p. injection of 4.0 meq of acetic acid, the mice were placed 10/cage in the incubator at 37°C. Other drugs administered at room temperature.
[c] Each 10 mice represents a single experiment.
[d] The subcutaneous route was used for these drugs. Others injected intraperitoneally (i.p.).
[e] Containing succinate, NaH_2PO_4 and Na_2HPO_4 equivalent to the amount injected with the prednisolone (Meticortelone), 40 mg/kg.

3.5. *Pathology*

Autopsy provided a clear dichotomy between those agents (acid or alkali) which were lethal and those which were not. However, mice killed at 37°C



(1) (2) (3)

Fig. 4. Animals 1 and 2 were injected intraperitoneally with acetic acid, 4 meq/kg, and placed at 23 and 37°C, respectively. Number 3 is a saline-treated control at 37°C. The mice were sacrificed 2–3 hr after the injections. Note the similarity of the reaction to the acid in animals number 1 and 2.

at spared at 23°C by the same agent were indistinguishable by the parameters employed.

Grossly, the animals given the lethal agents and kept at either 23°C or 37°C showed a marked congestion in the intestines and peritoneum, and the gut appeared shrunken and dark red (fig. 4). Straw-colored, fibrin-free fluid was found in the peritoneal cavity. The general appearance was of an acute inflammatory process. Gross hemorrhage was not evident, and the peritoneal fluid was only rarely blood-tinged. At most, only mild congestion was observed in animals given nonlethal agents, and peritoneal fluid was generally absent. Gross discoloration of intestines was not seen.

Microscopically, the congestion of intestines seen grossly was confirmed. However, peritonitis was ruled out, as actual inflammatory cells were scant (fig. 5). There was sloughing and necrosis of the mucosa. This indicates that the acid produced more than a topical irritating effect since the lesion was on the luminal side. The intestines of mice given nonlethal agents had normal-looking mucosa. No pathology was evident, except perhaps for some mild congestion.

The lethal agents all evoked an exudate which was 4–7 times greater in amount than the 0.1 mL of solution injected. Smears of this exudate revealed numerous polymorphonuclear leukocytes. The nonlethal agents invariably showed no fluid in the peritoneal cavity after 1–3 hr.

4. DISCUSSION

From the results with the various acids, bases and salts (table 1), we can conclude that (1) certain acids and bases, whether weak or strong, but not their salts have the potential for this toxicity; (2) the toxicity is influenced by the ambient temperature with a higher mortality for single mice in the cold than those housed in groups of 10; (3) the toxicity is dose-related with an LD_{50} of about 4 meq/kg for both weak and strong acids and bases; and (4) the toxicity is correlated with the potential hydrogen (or hydroxyl) ions present (equivalence) rather than with the measurable ion concentration (pH).

Though we came upon the phenomenon of intraperitoneal acid toxicity accidently, a similar observation had been made by Opie (1964) working with rats at room temperature. He found that only those agents which caused a peritoneal exudate were lethal, and that the rate of mortality was dose dependent. Thus, some of his observations in another species are similar to our own.

The central amine-depleting effect of reserpine and tetrabenazine and the associated depression seemed unlikely as the cause of death since we found no correlation between changes in brain norepinephrine and toxicity. Furthermore, chlorpromazine and phenobarbital which did not lower brain norepinephrine markedly depressed the animals but did not kill them. The former two drugs had been solubilized in acetic acid whereas the latter two were used as their water-soluble salts.

An attempt was made to rule out acidosis as a cause of death by the use of carbonic anhydrase inhibitors which produce a metabolic acidosis together with a marked increase in urinary bicarbonate (Maren, 1967). An increase in urinary pH and prevention of electroshock-induced seizures in the mice (Gray et al., 1958) were utilized as an indication of a drug effect. No deaths occurred with these agents at either 23°C or 37°C suggesting that at least under these conditions this type of acidosis was inocuous. It might be pointed out that acid loads larger than those used here are routinely used in dogs (Pitts, 1963). Injecting the acetic acid subcutaneously was not fatal to the mice at 37°C, but a rapid intravenous injection of the acid killed the mice in a few seconds. This was probably due to a direct cardiac effect of the acid.

An interesting parallel to the temperature depe[illegible]ent mortality is seen in the work of Berry (196[illegible]) who found that the LD_{50} of crude endotoxin [illegible] 37°C or 5°C environmental temperature in mice to [illegible] about 1/35 the LD_{50} at 25°C, and about 1/60 [illegible] dose at 30°C (temperature at which the mice w[illegible] most refractory to the endotoxin). In measuring bo[illegible] temperatures he found that the endotoxin apparen[illegible] produced a fatal hyper- or hypothermia. Our resu[illegible] are also similar to Berry's as regards time of de[illegible] (within 4 hr) and rapidity of temperature char[illegible] (within 30 min).

All of Berry's mice were housed one per cage. [illegible] our animals housed individually at 5°C, we h[illegible] observed a 100% death rate between 4 and 5 hr w[illegible] 4 meq/kg acetic i.p., whereas under these same co[illegible]tions no saline-treated controls or acid-treated a[illegible]mals housed 10/cage have died. Further, there wa[illegible] significantly lower ($p < 0.001$) temperature in th[illegible] animals housed singly at the end of 4 hr. Appare[illegible] the body heat insulated by grouping the mice at 5[illegible] was sufficient to spare them, whereas those hou[illegible] singly had no such protection and succumbed.

The pathophysiology of experimental endoto[illegible] death in mice has not been well characterized as [illegible] the dog (Lilichei et al., 1964) so that we could [illegible] rule out a primary toxic effect as occurs with [illegible] exogenous administration of endotoxin at room te[illegible]perature. Endotoxin, a complex lipopolysaccharide, [illegible] part of the gram-negative bacteria in the gut of th[illegible] animals. Perhaps intestinal endotoxin is released a[illegible] result of bacterial lysis secondary to the irritat[illegible] agent. Unexpectedly, the lesion proceeded from [illegible] lumen side of the intestine rather than from the s[illegible] of the peritoneal cavity. If there were an end[illegible] toxemia, it would explain the different death rates [illegible] well as the similar pathology found at 23 and 37[illegible] under similar conditions a specified amount of end[illegible] toxin is lethal only at the higher temperature (Ber[illegible] 1966).

The evidence for a role of endotoxin in t[illegible] mortality of these mice is indirect at present. If [illegible] endotoxin is in the future identified, an advantage [illegible] this model is that the substance is liberated fre[illegible] bacteria within the body. Thus, this method would [illegible] relatively free of the errors associated with the adm[illegible]istration of an artificial product. It should be no[illegible] that although antibiotics did not prevent death, t[illegible]

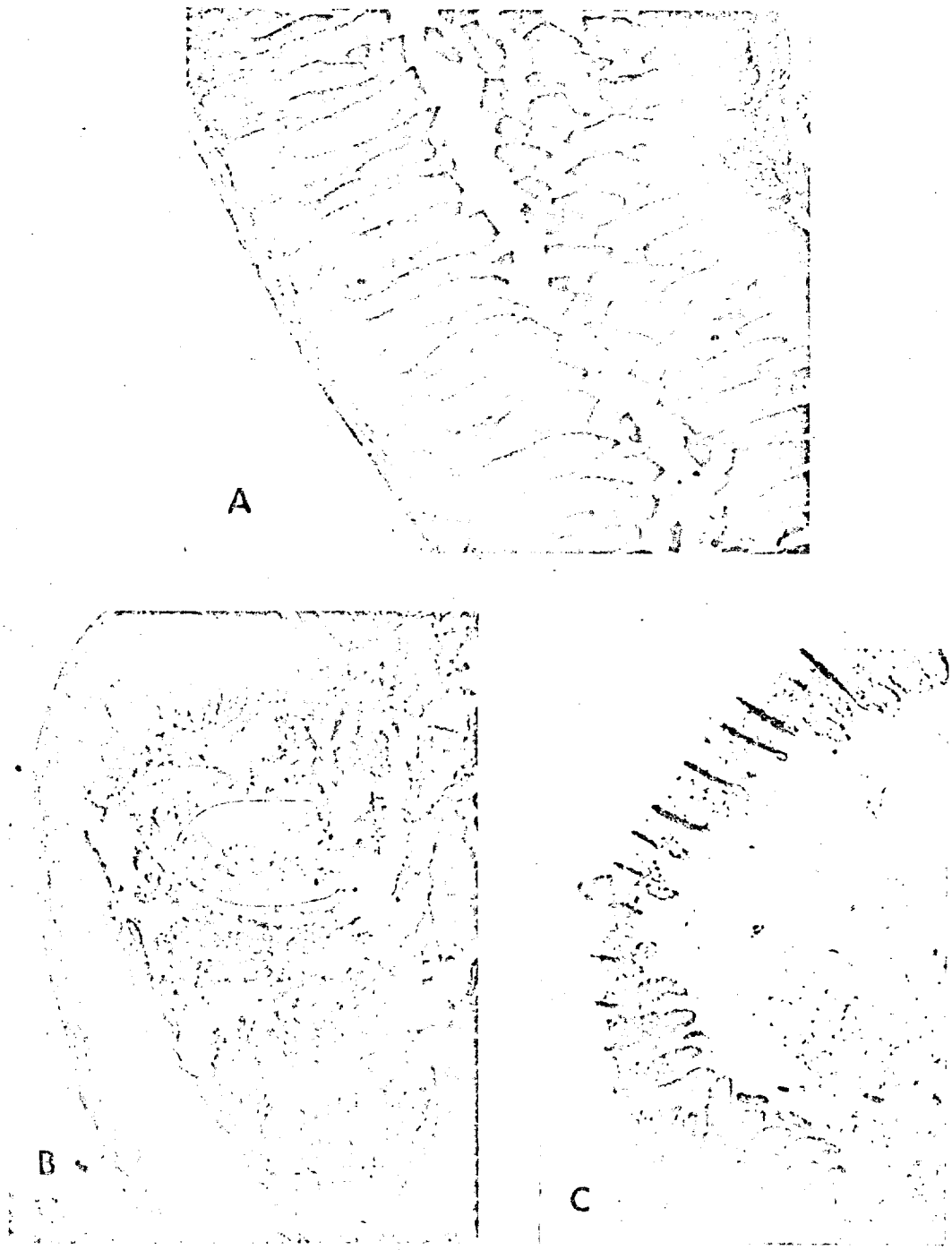


Fig. 5. Microscopic view of the intestinal mucosa from the mice treated the same as in fig. 4. A, saline-treated (100X); B and C (40X), acid-treated and placed at 23° and 37°C, respectively. Note sloughing and necrosis of the mucosa from the acid-treated mice.

s not indicate an extra-bacterial cause of mortality e no attempt was made to sterilize the gut.

Preliminary screening with various compounds ved that steroids might be beneficial (table 3). y have been shown to afford some protection nst the lethal effects of exogenous endotoxin ller et al., 1954). However, since protection was ifested only when the drug was given 5 min re the acid, we cannot dismiss a simple dilutional ct as the reason for protection. And yet the ending salt medium itself, without the steroid, ided no protection. Further studies with these ent drugs are in order to see if they really are eficial.

Glucose given i.p. protected, but had no effect en the same amount was given subcutaneously le 3). An equimolar amount of saline, and an volume of physiologic saline, both given intra-toneally, offered no protection. Glucose injected peritoneally is probably acting to osmotically fluid from the peritoneum and dilute the injected l.

Chlorpromazine, the β-adrenergic blocker propra-ol, and the α-adrenergic blocker phenoxybenz-ne have all been reported to be of benefit in rimental endotoxic shock (Berk et al., 1969; hei et al., 1964; Noyes et al., 1956). Though in work we did not find them beneficial (table 3), cies and other experimental variations preclude meaningful comparisons (Zweifach, 1961). mpts to prevent death with analgesics and local thetics, by obtunding any contributing pain, were unsuccessful.

Various modes of experimentally inducing a el-like state in animals have been used over the rs. In the mouse these include hemorrhagic, septic dotoxic, immunologic and traumatic (tourni-t, burn) methods (Zweifach, 1963). This work provide a useful method of producing a mortal-n mice that resembles endogenous endotoxemia. Simplicity and easy reproducibility make it con-ent for carrying out studies to elucidate the cause ath as well as its possible pharmacologic preven-n.

ACKNOWLEDGEMENT

The authors are indebted to Miss Ana Serrano for her capable technical assistance.

REFERENCES

Anton, A.H. and D.F. Sayre, 1962, A study of the factors affecting the aluminum oxide-trihydroxy indole procedure for the analysis of catecholamines, J. Pharmacol. 138, 260.

Berk, J.L., J.F. Hogen, W.H. Beyer, M.J. Gerber and G.R. Douchat, 1969, The treatment of endotoxin shock by beta-adrenergic blockade, Annals Surg. 169, 1.

Berry, I.J., 1966, Effect of environmental temperature on lethality of endotoxin and its effect on body temperature, Federation Proc. 25, 1264.

Geller, P., E.R. Merrill and E. Jawetz, 1954, Effects of cortisone and antibiotics on lethal action of endotoxin in mice, Proc. Soc. Exptl. Biol. Med. 86, 716.

Gray, W.D., C.E. Rauch, A.C. Osterberg and L.M. Lipchuck, 1958, The anti-convulsant actions of methazolamide (a carbonic anhydrase inhibitor) and diphenylhydantoin, J. Pharmacol. 124, 149.

Lillehei, R.C., J.K. Longerbeam, J.H. Bloch and W.G. Manax, 1964, The modern treatment of shock based on physiologic principles, Clin. Pharmacol. Therap. 5,63.

Maren, T.H., 1967, Carbonic anhydrase: chemistry, physiology, and inhibition, Physiol. Rev. 47, 595.

Noyes, H.E., J.P. Sanford and R.M. Nelson, 1956, Effect of chlorpromazine and dibenzyline on bacterial toxins, Proc. Soc. Exptl. Biol. Med. 92, 617.

Opie, E.L., 1964, Inflammation in serous cavities, Arch. Pathol. 78, 1.

Orlans, F.B.H., K.F. Linger and B.B. Brodie, 1960, Pharmacological consequences of the selective release of peripheral norepinephrine by syrosingopine (SU 3118), J. Pharmacol. 128, 131.

Pitts, R.F., 1968, Physiology of the kidney and body fluids, 2nd ed. (Year Book Publishers, Chicago).

Shore, P.A., 1962, Release of serotonin and catecholamines by drugs, Pharmacol. Reviews, 14, 531.

Zweifach, B.W., 1961, Aspects of comparative physiology of laboratory animals relative to the problems of experimental shock, Federation Proc. (Suppl.) 9, 18.

Zweifach, B.W., 1963, Annotated Bibliography on Shock 1950-1962, Publication 1182, National Academy of Sciences, National Research Council, Washington, D.C.

Biochem. J. (1967) 103, 400

Comparison of Oxidative Metabolism in Starved, Fat-Fed and Carbohydrate-Fed Rats

By P. A. MAYES* and J. M. FELTS
Cardiovascular Research Institute, University of California, School of Medicine, San Francisco, Calif., U.S.A.

Biochem. J. 103: 400-6 (1967)
(*Received* 2 *August* 1966)

1. Rats were starved for 48hr. or fed for 1 week on a high-fat or a high-carbohydrate diet. The effects of these dietary alterations on the rate of production of $^{14}CO_2$ from trace amounts of [U-^{14}C]glucose, [1-^{14}C]palmitate or [1-^{14}C]acetate administered intravenously were studied. 2. The oxidation of [^{14}C]glucose was most rapid in the carbohydrate-fed condition and was decreased significantly and to the same extent after starvation and after feeding with fat. 3. Under all dietary regimes studied the maximum rate of elimination of $^{14}CO_2$ from [^{14}C]palmitate occurred within a few minutes after injection, but considerably more was oxidized after starvation and feeding with fat than after feeding with carbohydrate. 4. Alterations in diet had no effect on the oxidation and high recovery of administered [^{14}C]acetate as $^{14}CO_2$. 5. Graphical analysis showed the presence of several exponential components in the $^{14}CO_2$-elimination curves. 6. In all studies a marked similarity in oxidative pattern was noted between the starved and the fat-fed rat.

Rats on a high-fat diet, on evisceration, maintain higher concentrations of blood glucose and survive twice as long as rats on a high-carbohydrate diet (Roberts, Samuels & Reinecke, 1943–44). Experiments *in vitro* (Masoro, Chaikoff, Chernick & Felts, 1950) have shown that either starvation or the feeding of a high-fat diet leads to lower recoveries of $^{14}CO_2$ when [^{14}C]glucose is oxidized by liver slices. Hansen, Ratter & Samuels (1951) found that glucose uptake is depressed in isolated diaphragms from both starved rats and rats that had received a high-fat diet when compared with the uptake by diaphragms from rats on a high-carbohydrate diet. These findings are compatible with a diabetic-type glucose-tolerance curve found in humans on diets low in carbohydrate (Himsworth, 1935). All of these results suggest that feeding with fat exerts a sparing action on carbohydrate utilization.

On the other hand animals on a high-carbohydrate diet or given a glucose load metabolize more glucose and spare fat. McCulla, Oates & Gordon (1957) found that giving glucose to starved rats decreased substantially the oxidation of injected [^{14}C]palmitate to $^{14}CO_2$. In the intact animal the fat-sparing action of carbohydrates is most prominent on the oxidation of the longer-chain fatty acids (Lossow & Chaikoff, 1955). Masoro & Felts (1958) demonstrated that the addition of glucose to liver slices from fed rats suppressed the oxidation of long-chain fatty acids but not the oxidation of the C_4–C_{10} fatty acids.

No systematic study has yet appeared comparing all of these interrelations *in vivo* with substrates administered intravenously. We have compared the effects of starvation, of a high-fat diet and of a high-carbohydrate diet on the oxidation of trace quantities of [^{14}C]glucose, [^{14}C]palmitate and [^{14}C]acetate injected intravenously into unanaesthetized intact rats. The $^{14}CO_2$-elimination curves were examined by graphical analysis, and the nature of the logarithmic components is discussed.

MATERIALS AND METHODS

Animals. Male Long Evans rats (180–210g.) were maintained on a stock diet (Diablo Laboratories, Berkeley, Calif., U.S.A.) and then transferred to individual cages and given a high-carbohydrate or a high-fat diet *ad libitum* for 1 week. The high-carbohydrate diet consisted of: vitamin-free casein, 5%; corn starch, 40%; glucose, 50%; salt mixture, 3% (Hubbell, Mendel & Wakeman, 1937); brewer's yeast, 2%; cod-liver oil 0·2%. In the high-fat diet, an isocaloric amount of lard fat was substituted for the starch and glucose. Each rat was handled frequently to accustom it to the future experimental procedure. Only those animals that maintained or increased their body weight on the experimental diets were used for study. Another group of rats, designated 'starved', was maintained on the stock diet and food was withdrawn 48hr. before the experiment. Drinking water was always available.

* Present address: Division of Biochemistry, Department of Physiology, Royal Veterinary College, London, N.W.1.

Preparation of substrates. Solutions of radioactive substrates were prepared to contain approx. 4μc in 1ml. [1-^{14}C]Acetate (1·53mc/m-mole; Research Specialties Co., Richmond, Calif., U.S.A.), [U-^{14}C]Glucose (2·9mc/m-mole; New England Nuclear Corp., Boston, Mass., U.S.A.) and $NaH^{14}CO_3$ (5mc/m-mole; New England Nuclear Corp.) were all dissolved in 0·9% NaCl solutions. [1-^{14}C]Palmitate (10mc/m-mole; New England Nuclear Corp.) was combined with bovine serum albumin (Armour Laboratories, Kankakee, Ill., U.S.A.) according to the method of Masoro & Felts (1958).

Experimental procedure. Each rat received, via a tail vein, approx. 2μc of a trace amount of substrate between 9·00 and 10·00a.m. Immediately after the injection, each rat was placed in a metabolism chamber constructed from polyethylene bottles of total volume 800ml. Room air was drawn continuously through the chamber at a rate of 450ml./min. and bubbled through two CO_2 absorbers connected in series; each absorber contained 50ml. of 2N-NaOH. At 5, 15, 30, 60, 90, 120, 150 and 180min. after injection, the contents of the two absorbers were removed quantitatively and replaced with fresh 2N-NaOH. The contents and washings were combined and diluted to 200ml. Samples of the NaOH carbonate solutions were analysed for radioactivity. After 3hr. the rats were decapitated and their blood was collected in heparinized tubes.

The results are expressed as percentages of dose expired/min. at 2·5, 10, 22·5, 45, 75, 105, 135 and 165min., these values being calculated from the amounts of $^{14}CO_2$ collected during each time-interval.

Analytical methods. Radioactive carbonate was estimated by adding 5·0ml. of the NaOH–carbonate solution and 10·0ml. (3ml.) to the outer compartment of a 50ml. Erlenmeyer flask fitted with a centre well containing methylamine (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.) in methanol (0·5ml.). The flask was stoppered with a rubber serum-bottle stopper and 5ml. of air was withdrawn by syringe and needle, and 1·0ml. of N-H_2SO_4 was injected into the outer compartment. After shaking for 1hr. at room temperature, the contents of the centre well were transferred quantitatively with washings of methanol into a conical vial. The methanol was evaporated under a stream of air and the residue was dissolved in toluene containing 0·1% of 2,5-diphenyloxazole and 0·03% of 1,4-bis(5-phenyloxazol-2-yl)benzene. The samples were counted in a liquid-scintillation spectrometer (Packard). The counts were corrected for quenching by using an internal standard.

Blood glucose was estimated by a glucose-oxidase method (glucostat reagent; Worthington Biochemicals Corp., Freehold, N.J., U.S.A.). Plasma free fatty acids were determined by the Dole procedure by using the modification of Trout, Estes & Friedberg (1960).

Statistical tests were carried out by analysis of variance. $P<0·05$ was taken as the criterion of statistical significance. Variations are shown as the S.E.M.

RESULTS

The rate of expiration and the total recovery of $^{14}CO_2$ in 3hr. after the intravenous administration of ^{14}C-labelled substrates is shown in Fig. 1. The elimination patterns of $^{14}CO_2$ of each substrate tested were similar in starved and fat-fed rats. However, apart from the results with [1-^{14}C]acetate, there were marked differences between the patterns found in carbohydrate-fed rats on the one hand and those in starved or fat-fed rats on the other.

[U-^{14}C]Glucose. In 3hr. there was no statistical difference in the mean recoveries of $^{14}CO_2$ between the starved (34·4±0·6%) and fat-fed (36·6±2·6%) groups of rats. In the starved rats the maximum rate of elimination of $^{14}CO_2$ was found at 60min. after injection of the dose, whereas in the fat-fed rats it was found after 45min. These results differed significantly from those obtained when [^{14}C]glucose was administered to carbohydrate-fed rats. In these experiments 53·5±2·3% of the dose was recovered and the maximum rate of elimination of $^{14}CO_2$ occurred only 15min. after injection.

[1-^{14}C]Palmitate. Similarities between starved and fat-fed rats were evident in this series of experiments. In 3hr. there was a mean recovery of administered ^{14}C as $^{14}CO_2$ of 51·4±2·4% in the starved animals and 50·0±4·9% in those on the fat diet; the corresponding value for the carbohydrate-fed rats was only 32·2±2·4%. Under all dietary conditions, the maximum rate of elimination of $^{14}CO_2$ occurred very soon after injection, within 5min. in the starved and fat-fed animals and within 10min. in those on the carbohydrate diet.

[1-^{14}C]Acetate. Mean recoveries of $^{14}CO_2$ of 79·8±1·6%, 79·0±0·8% and 80·2±0·6% were obtained in 3hr. from carbohydrate-fed, fat-fed and starved rats respectively. Irrespective of dietary treatment, the maximum rate of expiration of $^{14}CO_2$ occurred within 5min. of injection.

The values shown on the curves in Fig. 1 were plotted on semilogarithmic co-ordinates in Fig. 2, and the method of graphical analysis of the curves into their components was adopted (Riggs, 1963). Only the descending portion of each curve has been treated in this manner.

In each experiment in which [U-^{14}C]glucose was administered, only a single negative exponential function was evident, which had half-times of 49, 71 and 123min. in carbohydrate-fed, fat-fed and starved rats respectively. However, the semilogarithmic plots of the results from the rats given [^{14}C]acetate or [^{14}C]palmitate indicated several exponential functions. After the administration of [^{14}C]acetate, two components having half-times of approx. 14 and 70min. were demonstrated under all dietary conditions. After the administration of [^{14}C]palmitate there were three components in starved and fat-fed rats, but only two in carbohydrate-fed rats. The slowest component had a similar slope on all dietary treatments, ranging from a half-time of 165min. in carbohydrate-fed animals to 200min. in those fed with fat. The half-times of the intermediate component found in the fat-fed and starved rats were 21 and

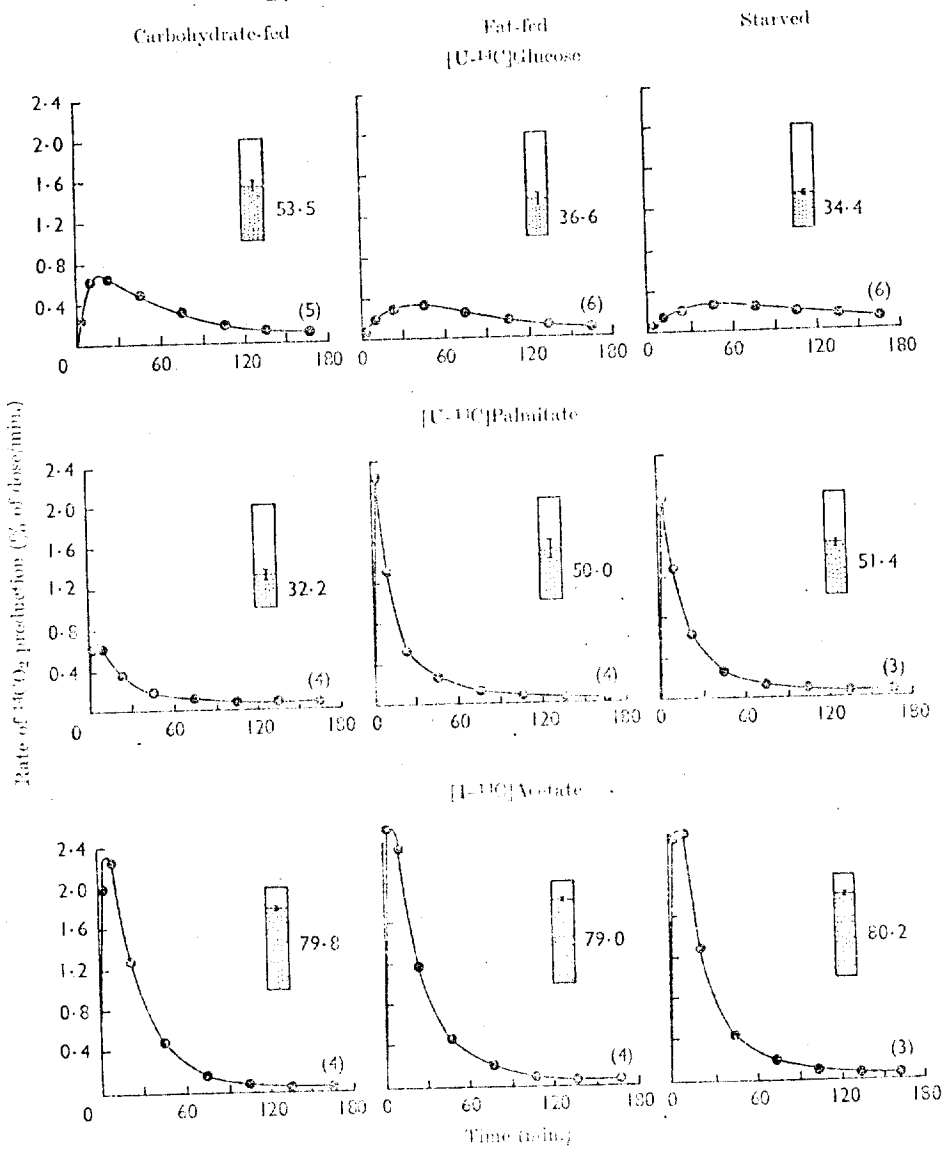


Fig. 1. Rate of elimination of $^{14}CO_2$ from intact rats given ^{14}C-labelled substrates intravenously. The percentage recovery in 3hr. of radioactivity administered is shown in the stippled rectangle. The number of rats in each group is shown in parenthesis. Variations indicated are the S.E.M.

22·5min. respectively. The half-times of the initial component were only 6·5min. in the fat-fed rats, 9·5min. in the starved rats and 14min. in the carbohydrate-fed rats.

Graphical analysis of the results obtained from both fed and starved rats given [^{14}C]bicarbonate (Fig. 3) showed three exponential components having half-times of 6, 15 and 62 min. in the fed animals and 6·5, 18·5 and 52·5min. in starved animals. A separate experiment showed that the half-time of washout of $^{14}CO_2$ from the metabolistic characteristics was 2·9min.

Analysis of the blood samples obtained from the rats after the 3hr. period of carbon dioxide collec-

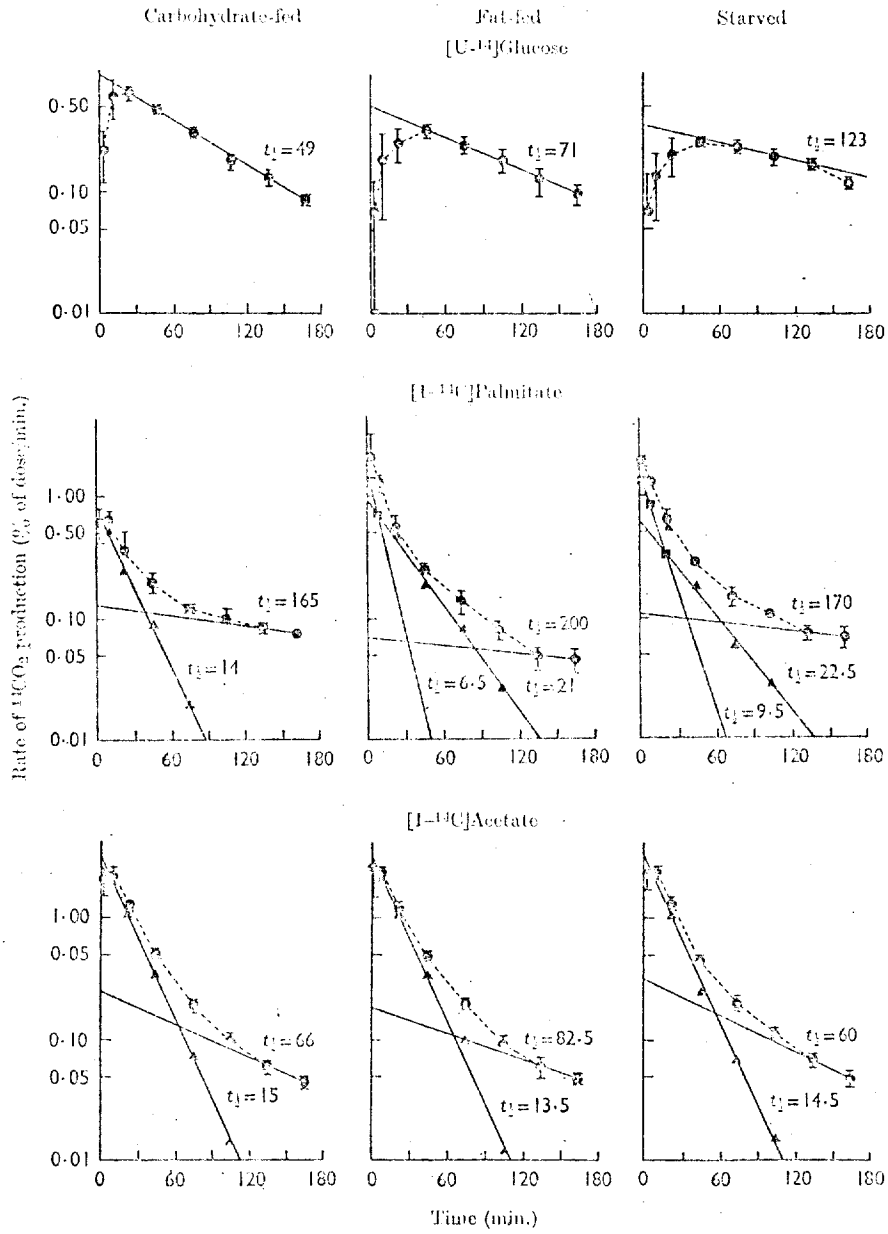


Fig. 2. Rate of elimination of $^{14}CO_2$ from intact rats given ^{14}C-labelled substrates intravenously. The curves are analysed graphically into their exponential components (see the text). The S.E.M. values are indicated by the bars on the experimental curve.

have the following results: Mean plasma free fatty acid concentrations in carbohydrate-fed, fat-fed and starved rats were 0·50 ± 0·01, 0·46 ± 0·03 and 0·60 ± 0·03 m-equiv./l. respectively. The corresponding values for the blood glucose concentrations were 110 ± 2·1, 99 ± 4·3 and 93 ± 2·9 mg./100 ml.

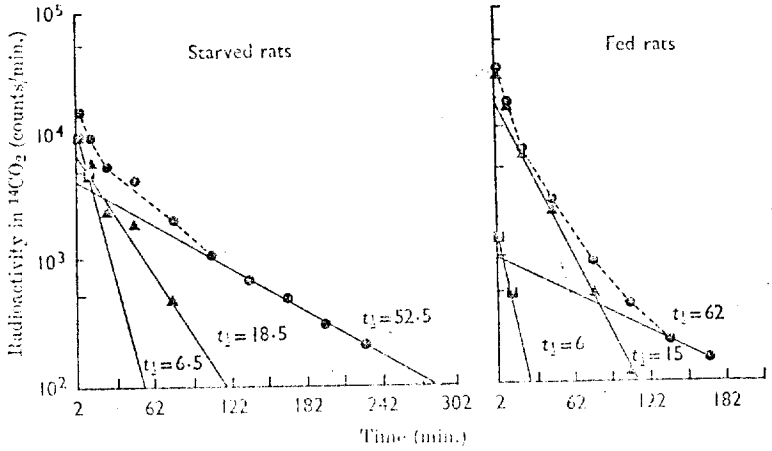


Fig. 3. Rate of elimination of $^{14}CO_2$ from fed or starved rats given a single intravenous injection of $NaH^{14}CO_3$ at zero time. The curves are analysed graphically into their exponential components (see the text). There were two animals in each group.

DISCUSSION

We have compared the effects of a high carbohydrate diet, of a high-fat diet and of starvation on the rates of oxidation of several labelled substrates administered intravenously in the intact rat. When substrates have been administered in unphysiological preparations, or have been given by stomach tube or intraperitoneally, there have always been considerable delays in oxidation compared with the results reported here. In the present study, the rats were conditioned to the experimental procedures, so that no undue alteration in pool size of either glucose or free fatty acids would take place on handling. No attempt was made to follow plasma specific activity for any of the substrates injected, since the turnover times of two of these, acetate and palmitate, are too rapid to be meaningful when related to radioactivity in expired carbon dioxide. Thus we have measured the rate at which $^{14}CO_2$ was expired but not the total production of carbon dioxide.

Rats maintained on the high-carbohydrate diet oxidized the [^{14}C]glucose administered faster than did the starved animals or the animals previously given the high-fat diet. The blood glucose concentration at the end of the experimental period showed little variation between the three groups of rats, indicating a corresponding lack of variation in the size of the extracellular glucose pool. The results of previous work (Mayes, 1963*a*,*b*) indicate that, during the experimental period, the blood glucose concentration probably remained fairly constant in all rats, and that the initial liver glycogen content (with which the label might equilibrate) was probably highest in the carbohydrate-fed rats. The marked decrease in the rate of oxidation of labelled glucose in the starved and fat-fed rats is therefore minimized rather than exaggerated by any changes in pool size. The observation that the maximum rate of elimination was not reached until 15min. in the carbohydrate-fed rats indicates that some time is required for intermediate pools to reach maximum specific activity. This delay was much longer in the starved and the fat-fed rats, implying that the rate of transfer of label through the intermediate pools was depressed. This may have resulted from the inhibition of certain key enzymes, e.g. phosphofructokinase (Randle, Garland, Hales & Newsholme, 1963), involved in glucose oxidation. The delay also implies a much lower oxidative turnover rate of blood glucose in both the starved and the fat-fed rats. Baker, Shipley, Clark, Incefy & Skinner (1961) have discussed the significance of the time of occurrence of maximum $^{14}CO_2$ specific activity. Our data emphasize the similarity in the pattern of oxidation of glucose in the starved and the fat-fed animal.

Starved rats or rats maintained on a high-fat diet oxidized [^{14}C]palmitate more quickly than did carbohydrate-fed animals. As free fatty acids are combined with albumin in the plasma, the pool into which the labelled palmitate was injected was probably confined to the plasma albumin space. The concentrations of plasma free fatty acid were not markedly different among the groups of rats at the end of the 3hr. period in the metabolic

[illegible]. However, it is likely (see Bergstrom, 1962a) [illegible] the rate of oxidation of the [illegible] fatty acids, and this would account for the [illegible] and in the rate of oxidation of [illegible]palmitate between the various nutritional states. Unlike the results with [illegible]-glucose, the maximum rate of production of $^{14}CO_2$ from [illegible]-palmitate was reached within a few minutes of injection, implying that plasma free fatty acid have a very rapid access to the oxidative machinery. In carbohydrate-fed animals the maximum activity was not reached as rapidly as in the other states that were observed in this fast. This might be due to an increase in activity of enzymes concerned in the utilization of free fatty acids. In addition, a decrease in oxidation of the [illegible]palmitate administered was apparent for the starved and fed states of rats. This mechanism is not affected by dietary [illegible] control the rates of oxidation of both glucose and long-chain fatty acids.

No control of the oxidation of [illegible]-palmitate by variations in the diet was encountered. The possible [illegible] for this are discussed below.

The $^{14}CO_2$-elimination curves from the radioactive [illegible] have been resolved into three exponential components (Fig. 2). Both the stages, or the number of components, may be influenced by the labelling of the plasma bicarbonate pool and by the washout characteristics of the metabolism chamber. The very short half-time (0·9 min.) observed for the $^{14}CO_2$ washout from the metabolism chamber shows that this would not be a significant factor affecting the interpretation of the $^{14}CO_2$-elimination data from rats. In the experiments where [illegible]-bicarbonate was injected, 2 min. was allowed to elapse before beginning the collection of $^{14}CO_2$ to allow time for equilibration of the labelled [illegible] circulation (Drury, Wick & Abson, 1950). The order of components and the rate of elimination of $^{14}CO_2$ from all animals given [illegible]-bicarbonate were similar. Analysis of the $^{14}CO_2$-elimination curves by graphical means led to the consideration of three exponential components (Fig. 3), in [illegible] agreement with Shipley, Baker, Brody & [illegible] (1958), who used the single-injection procedure, and with Morris & Thompson-Morgan (1963), who used a continuous-infusion technique. In [illegible] with the present work, these studies showed an exponential component having a half-time of approx. 6 min. which is likely to represent [illegible] from the pool of bicarbonate in extracellular fluid (Shipley *et al.* 1959). The curve of the intermediate half-time is probably due to elimination of $^{14}CO_2$ once equilibrium has been reached in the microcellular bicarbonate pool. The third component may represent the elimination of $^{14}CO_2$ by various tissues of slow turnover.

It is proposed that whenever the rate of oxidation of a substrate has a half-time shorter than the time required for the intra- and extracellular bicarbonate pools to attain equilibrium, this will result in three main components of $^{14}CO_2$ elimination, owing to a rapid labelling of the extracellular bicarbonate pool. If the rate of oxidation is lower, resulting in an initial component of intermediate half-time, only two components will result since adequate time is available for the intra- and extracellular bicarbonate pools to equilibrate to form one effective pool. Correspondingly, if the rate of oxidation results in a component having a long half-time, then only one component might be expected as the intra-bicarbonate pool would be turning over as fast as or faster than that of the substrate undergoing oxidation.

The data of Baker, Brown & Shipley (1956) suggest only a single exponential component in the $^{14}CO_2$-elimination curve obtained from the oxidation of [illegible]-glucose. Our results show also an apparent single exponential component under a wide range of dietary variations. The interrelations between the bicarbonate pool and the glucose pool have been considered by Baker *et al.* (1961).

Amplified analysis of the $^{14}CO_2$-elimination curves from [illegible]-palmitate yielded three exponential components from the starved and fed rats, but one of these components was not present after feeding with carbohydrate. When [illegible]-palmitate was administered to fed rats, the third component had a very short half-time of 0·5 min. This indicates that this [illegible]-palmitate was metabolized extensively, producing a rapid equilibration of the bicarbonate pool. The fact that the amount of the initial component from the starved rat was fed animals would also indicate a delay in the time at which the maximum rate of $^{14}CO_2$ oxidation occurred (Fig. 1). The possible reasons for the delay have been discussed.

The mechanism whereby carbohydrate inhibits the oxidation of long-chain fatty acids, which has been reviewed by Fritz (1963), was considered to be a result of the disadvantageous action of citrate on the activation of acyl-coenzyme A formation in adipose tissue extracts. The mechanism whereby fatty acids inhibit the oxidation of glucose has also received attention. Randle *et al.* (1963) have suggested on the basis of observations that fatty acids diminish the uptake of glucose, decrease glycogen synthesis by inhibiting phosphofructokinase and impair pyruvate oxidation in tissues. These explanations could account for our observations on the inhibition of glucose oxidation during starvation or the alloxan diabetic state, when fat, when the concentrations of free fatty acids are elevated.

The analysis of the $^{14}CO_2$-elimination curves after the injection of [illegible]-acetate revealed two

exponential components that were not influenced by the dietary state. In the fat-fed and starved rats, the rate at which $^{14}CO_2$ was eliminated fell more slowly immediately after the administration of [^{14}C]acetate than after the administration of [^{14}C]palmitate. An explanation of this may be that the specific activity of [^{14}C]acetate remains high for a longer period after injection owing to its distribution into a fluid compartment considerably larger than the albumin space into which [^{14}C]palmitate would equilibrate. This may obscure any alterations of oxidative patterns caused by changes in the diet. Thus, when trace amounts of acetate are injected, it appears that the rate of evolution of $^{14}CO_2$ does not give any information on metabolic processes that are very rapid, such as the comparative turnover times of the citric acid cycle.

It is difficult to explain the results showing undiminished total oxidation of [^{14}C]acetate in carbohydrate-fed animals. It would be expected that more label would be diverted into lipogenic pathways under these conditions, thereby lowering the recovery of label expired as $^{14}CO_2$. It is possible that the rats were not studied during that period of the feeding cycle when lipogenesis was most active.

The results show how similarities exist in the metabolic patterns of the fat-fed and the starved animal. In both dietary states there is an apparent sparing of [^{14}C]-glucose oxidation and an enhancement of the oxidation of [^{14}C]palmitate. Similar exponential components are found in the $^{14}CO_2$-elimination patterns. These findings support the postulate (Felts & Mayes, 1967; Felts, 1965; Mayes & Felts, 1966) that the major pathway of assimilation of dietary fat into the tissues involves the prior hydrolysis of triglyceride to free fatty acid. The similarity in metabolic pattern of the starved and the fat-fed rat is due most probably to free fatty acids being the primary metabolic fuel in both conditions.

The authors thank Miss Margaret O'Hagen, Miss Tazuko Hirai and Mr R. McClendon for technical assistance. The work was supported by Grant HE-06285 of the U.S. Public Health Service. P. A. M. acknowledges receipt of a Wellcome Trust Research Travel Grant and J. M. F. a Career Development Award of the National Heart Institute.

REFERENCES

Baker, N., Shipley, R. A., Clark, R. E., Incefy, G. E. & Skinner, S. S. (1961). *Amer. J. Physiol.* **200**, 863.

Drury, D. R., Wick, A. N. & Almen, M. C. (1956). *Amer. J. Physiol.* **186**, 361.

Feller, D. D., Strisower, E. H. & Chaikoff, I. L. (1950). *J. biol. Chem.* **187**, 571.

Felts, J. M. (1965). *Ann. N.Y. Acad. Sci.* **131**, 24.

Felts, J. M. & Mayes, P. A. (1965). *Nature, Lond.*, **206**, 695.

Fritz, I. B. (1961). *Physiol. Rev.* **41**, 52.

Hansen, R. G., Ritter, W. J. & Samuels, L. T. (1951). *J. biol. Chem.* **192**, 215.

Hinsworth, E. P. (1935). *Clin. Sci.* **2**, 67.

Hubbell, R. B., Mendel, L. B. & Wakeman, A. J. (1937). *J. Nutr.* **14**, 273.

Lossow, W. J. & Chaikoff, I. L. (1955). *Arch. Biochem. Biophys.* **57**, 23.

McCaffa, C., Gates, H. S., jun. & Gordon, R. S., jun. (1957). *Arch. Biochem. Biophys.* **71**, 346.

Masoro, E. J., Chaikoff, I. L., Chetnick, S. S. & Felts, J. M. (1959). *J. biol. Chem.* **235**, 845.

Masoro, E. J. & Felts, J. M. (1958). *J. biol. Chem.* **231**, 317.

Mayes, P. A. (1962*a*). *Nature, Lond.*, **195**, 1071.

Mayes, P. A. (1962*b*). *Metabolism*, **11**, 781.

Mayes, P. A. & Felts, J. M. (1966). *Biochem. J.* **99**, 42c.

Morris, B. & Shapson-Morgan, M. W. (1962). *J. Physiol.* **169**, 543.

Randle, P. J., Garland, P. B., Hales, C. N. & Newsholme, E. A. (1963). *Lancet*, **i**, 785.

Rigas, D. S. (1963). *The Mathematical Approach to Physiological Problems*, pp. 120–167. Baltimore: The Williams and Wilkins Co.

Roberts, S., Samuels, L. T. & Reinecke, R. M. (1943–46). *Amer. J. Physiol.* **140**, 639.

Shipley, R. A., Fisher, N., Incefy, G. E. & Clark, R. E. (1959). *Amer. J. Physiol.* **197**, 41.

Trout, D. L., Estes, E. H. & Friedberg, S. J. (1960). *J. Lipid Res.* **1**, 199.

Life Sciences 7(2): 769-775, (1968)

UTILIZATION OF RUMEN METABOLITES AS ENERGY SOURCES IN RATS[1]

J. W. McAtee, C. O. Little and G. E. Mitchell, Jr.

Department of Animal Sciences, College of Agriculture
University of Kentucky, Lexington, Kentucky

(Received 9 April 1968; in final form 24 May 1968)

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 feeding 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. Female 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 1.0 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

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)

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 g or in crude protein, the carcasses of rats fed butyrate in experiment I contained more fat than did the carcasses of rats fed other diets. While slightly 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.

Slomter (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	5.76	-----	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.

References

1. ANDERSON, E. I. and D. LEWIS, Metabolism in the Rumen. John Wiley and Sons, Inc., New York (1959).
2. ARMSTRONG, D. G. and K. L. BLAXTER, Brit. J. Nutr. 11, 247 (1957).
3. ARMSTRONG, D. G., and K. L. BLAXTER, Brit. J. Nutr. 11,413 (1957).
4. BLIFIS, C. G. and G. E. MITCHELL, JR., Life Sciences 4,507 (1965).
5. BORISOFF, L. W. and F. V. HERSCHBERGER, Proc. Soc. Expt. Biol. and Med. 111, 973 (1963).
6. DUNCAN, D. B., Biometrics 11, 1 (1955).
7. N. R. C., Nutrient Requirements of Laboratory Animals. National Research Council, Washington, D. C. (1962).
8. [illegible], T. D., C. B. ELDER and O. T. GARRETT, J. Dairy Sci. 46, 13 (1963).
9. GLAZIER, E. L., The Energy Metabolism of Ruminants. C. C. Thomas, Publisher, Springfield, Ill. (1962).
10. GREEN, G. D. and B. W. AGRANOFF, Proc. Soc. Expt. Biol. Med. 114, 600 (1963).
11. HOGUE, D. E. and J. M. ELLIOT, J. Nutr. 83, 171 (1964).
12. SPOERSTAD, E. L. R., R. E. WEBB and ELLEN SHAH, J. Nutr. 88, 225 (1966).
13. VENKATARAMAN, S., D. K. BISWAS and B. C. JOHNSON, J. Nutr. 93, 131 (1967).
14. GUNNANI, S., P. MISTRY and B. C. JOHNSON, Biochem. Biophys. Acta 38,187 (1960)

Gastroenterology 5(5): 418-425, (1945)

ON THE PHYSIOLOGY OF VOLATILE FATTY ACIDS

J. F. McClendon, Ph.D., and John Scott, Ph.D.
Laboratories of Physiology, Hahnemann and Jefferson Medical Colleges, Philadelphia

INTRODUCTION

It was suggested by McClendon (1) that the volatile fatty acids arising from fermentation of roughage in the gut increase intestinal motility. It would be correct to speak of sodium salts of volatile fatty acids since enough sodium bicarbonate is secreted in the pancreatic and intestinal juices to neutralize these acids.

Grove, Olmsted and Koenig (2) showed that "high carbohydrate diets" increased volatile fatty acids in the stools, but their "high sugar diets" contained fruits and their "high starch diets" potatoes, both of which contain roughage. Olmsted, Curtis and Timm (3) concluded that whereas more than 80% of the hemicellulose from bran disappeared in the human gut it was not laxative nor did it increase the volatile fatty acids of the stools, whereas bran-cellulose was laxative and increased the volatile fatty acids of the stools. These results on purified cellulose and hemicellulose from bran might not seem easy to reconcile with those of Williams and Olmsted (4) who observed that the volume and volatile fatty acids of the feces are increased more by the amount of cellulose and hemicellulose that disappear than by the amount in the feces.

In studies of protein economy, Rubner and others showed that whereas bran proteins are to a large extent utilized, the eating of bran causes an increase of mucin and bacterial protein in the feces; hence one might say that bran increases secretion or synthesis of unabsorbable products that increase the bulk of the feces. It seems desirable to separate these various factors and the present experiments relate to the effect of volatile fatty acids on intestinal motility, particularly peristaltic waves.

Fraser (5) showed that hydrogen ions inhibit motor activity of the excised gut and this may explain the observation of Dunham (6) that 0.36-0.6 grams of acetic acid per kilogram body weight added to the baby's milk sometimes caused constipation. This amount of acid in addition to that in the gastric juice may have occasionally been greater than could be neutralized by the pancreatic and intestinal juices. Morgan and Thomas (7) observed that whereas acid in the duodenum stimulated it, inhibition of the stomach resulted, but whatever view is taken it seems best to rule out hydrogen ions when studying acetate.

The production of acetic and butyric acids in the human gut has been determined by several investigators. Macfadyen, Nencki and Sieber (8) found

12.5 millimols of acetic acid per kilogram of contents of the lower human ileum. Williams and Olmsted (4) found 13.52 millimols of volatile fatty acid in stools when 62.9 grams of cellulose and hemicellulose had fermented (failed to be recovered). Bergeim, Hanszen, Pincussen and Weiss (9) found 58 millimols of acetic and 58 millimols of butyric acid in the fluid voided after giving an enema.

EXPERIMENTS

In the following experiments whereas stimulation of the gut was produced by less than a millimol of sodium acetate the threshold of stimulation was not considered as important as the certainty that it was due to the acetate or butyrate ions. The solutions were made of the same osmotic pressure (0.5 *N*) pH (7.4) and temperature (38°) as the blood and one ion of calcium was present for every 100 ions of sodium. Gunn and Underhill (10) showed that the rate of intestinal contractions of any segment of the gut was a fixed quantity. The rate was always recorded but not observed to change in our experiments.

Excised gut

Sodium acetate or butyrate displacing 25 per cent of the Ringer-Locke solution containing rat or rabbit gut increased the amplitude of the rhythmic contractions of both longitudinal and circular muscle. The latter was measured by the method of Fraser (5). Acetic and butyric acids decreased the amplitude of contractions.

An attempt was made to apply the acetate to the mucosal side by turning the gut inside-out. Turning the gut inside-out abolished the rhythmic contractions of the longitudinal muscle. This is apparently due to the relief of tension in a circular direction on the peritoneal and longitudinal muscle layers (or myenteric plexus) of the wall. If a segment of gut is cut longitudinally it partially turns itself inside-out and there is a corresponding depression of motor activity. If a normal segment and an inside-out segment are cut longitudinally the motor activities of the two are the same. If a cut segment is sewed up its activity returns to the level before cutting.

In order to apply the acetate to the mucosal side without stimulation by increasing distension, an apparatus similar to that used by Polansky (11) was used. To avoid decomposition of the glucose it was added to the Ringer-Locke solution immediately before use. Acetate or butyrate on the mucosal side greatly increased the amplitude of the rhythmic contractions of all segments of rabbit and rat intestine. The effect seemed to be the same as on the peritoneal side but in the latter case the acetate and butyrate had access

to the cut ends and may have acted directly on the myenteric plexus. Since Polansky had observed that hydrogen ions inhibited the gut, experiments with the free acids were not repeated.

Experiments were made with the intestines of 40 rats and 10 rabbits. Each segment of the gut (duodenum, middle ileum, lower ileum and colon) had its characteristic rhythmic contractions and tonus waves, but in all cases the amplitude of the contractions was increased. After a segment had been contracting in Ringer-Locke solution for about 6 hours the rhythmic contractions might cease, in which case acetate or butyrate increased or initiated tonic contractions.

Experiments on the gut in situ

Experiments were performed by the balloon method on 25 dogs with Thiry-Vella or Biebl loops or under barbital anesthesia. Balloons were inserted

TABLE 1

Volatile fatty acid concentration (normality)

VESSEL	MINUTES AFTER INJECTION OF 100 CC. 0.2 *N* SODIUM ACETATE INTO DUODENUM							
	0	4	12	24	50	100	120	160
Portal vein	.0003	.0021	.0013	.0011	.0004	.0004	.0004	.0003
Iliac artery	.0003		.0004	.0004	.0003			
Iliac vein	.0003				.0003	.0003	.0003	
Hepatic vein	.0003							.0003
Intestinal content	.2000							.0080

into Thiry-Vella loops or held against Biebl exteriorized loops by means of a cuff. The introduction of acetate into the stomach was followed by vomiting or defecation. Considerable disturbance of the balloon record resulted from introducing the acetate into the stomach and its immediate effect was not recorded.

Under barbital anesthesia the balloon was placed in the duodenum and distended with water at 38°C. to 225 mm. water pressure, and the acetate was introduced directly into the intestine but it was thought that the anesthetic depressed intestinal contractions.

The absorption of the acetate into the blood was determined. The basal level of volatile fatty acid (VFA) of the blood is 0.0003 *N*, McClendon (12). When acetate was introduced into the gut it was absorbed and raised the VFA in the portal vein, but the liver removed most of it and the other tissues the remainder (table 1).

The intestinal content was 100 cc. of 0.2 *N* at the start and 18 cc. of 0.008 *N* at the end, showing 99.3% absorption. Similar results of acetate absorption had been observed by Barcroft, McAnally and Phillipson (13).

The VFA in the portal vein remained higher than basal for 120 minutes after injecting the acetate into the duodenum but had returned to the basal level in 160 minutes after 99.3% of the acetate had been absorbed. The arterial VFA was only slightly raised and returned to the basal level within 50 minutes (table 1).

Only by injecting relatively large quantities of acetate directly into the blood stream beyond the liver was it possible to raise the VFA in the iliac vein. Assuming that acetate acts as a building stone of acetyl-choline and that the necessary supply comes from the gut, we starved dogs 6 days, but this did not seem to alter the results of balloon experiments. If acetate is a building stone of acetyl-choline there is probably sufficient formed in the intermediary metabolism.

When the acetate was injected into the right iliac vein at the rate of 2.5 CC. of 0.2 *N* per minute, the VFA of the left iliac vein rose to 0.0004 *N*. When the rate was 7 cc. per min. the VFA was 0.0007, at 8 cc. per min. 0.0009, at 14 cc. per min. 0.0038 *N*. The motor activity of the duodenum paralleled the VFA in the blood.

Experiments with a double balloon

Twelve experiments with acetate and 3 with butyrate were made on 3 dogs with permanent gastric and duodenal fistulae by the method of Thomas, Crider and Morgan (14). The dogs were fasted 18 hours and hair balls removed from the stomach through the gastric fistula. A 3-lumen rubber tube bearing 2 balloons whose centers were 7 cm. apart was introduced into the lower duodenum with the dog in a standing position so that respiratory waves were not recorded. Rarely were pulse waves seen. The emptiness of the stomach and distance from pylorus was such that rarely was an effect of the stomach on the duodenum seen, Thomas and Crider (15). Each balloon was distended with 5 cc. of air and connected with a U-shaped water manometer with float bearing a writing point. About an hour was allowed for the gut to quiet down after the stimulus of distension. Five cc. of acetate or butyrate were introduced just oral to the oral balloon after it was shown that 5 cc. of Ringer's fluid introduced at the same rate had no effect on motor activity (tables 2 and 3).

In table 2 is given the effect of injecting 5 cc. of acetate: 3rd column the rhythmic contractions per minute (they were not changed by injecting acetate), 4th column the seconds elapsing between injecting acetate and increased pressure in oral balloon, 5th column ditto for aboral balloon and 6th to 9th

the increase in pressure. The highest pressure during any one rhythmic contraction is designated "systolic" and the lowest pressure "diastolic." The "diastolic" pressure before introducing acetate was often zero as nearly as could be determined. It may be seen that in all but the first experiment increased pressure occurred in the oral balloon before it did in the aboral, indicating a peristaltic wave. The delay between injection and increased

TABLE 2

Acetate

DOG. NO.	DATE	RHYTHMIC CONTRACTIONS PER MIN.	LATENT PERIOD IN SECONDS		INCREASED PRESSURE IN CM. H_2O			
					Oral		Aboral	
			Oral	Aboral	"Systolic"	"Diastolic"	"Systolic"	"Diastolic"
1	Mar. 8	19	40	40	14	6	14	6
	28	19	15	50	20	6	14	6
	29	19	20	40	15	4	15	6
2	Feb. 7	19	40	60	12	6	10	4
	21	19	15	60	13	4	17	4
	22	19	15	60	13	4	17	4
3	Mar. 1	19	60	120	16	10	16	8
	7	19	55	180	14	8	12	4
	8	19	70	250	12	6	10	6
	18	19	100	195	15	6	13	6
	28	18	25	40	15	8	12	6
	29	18	80	105	16	6	14	8
Average					14.5	6.2	13.7	5.6

TABLE 3

Butyrate (dog no. 3)

DATE	RHYTHMIC CONTRACTIONS PER MIN.	LATENT PERIOD IN SECONDS		INCREASED PRESSURE IN CM. H_2O			
				Oral		Aboral	
		Oral	Aboral	"Systolic"	"Diastolic"	"Systolic"	"Diastolic"
Mar. 9	19	60	120	14	8	10	4
14	19	80	120	7	4	14	6
15	18	200	200	7	4	14	8

pressure in the oral balloon was probably the time required for the wave to pass from the region of injection to the center of the oral balloon. In agreement with Alvarez (16) the Bayliss and Starling "law of the intestine" was observed in only the minority of cases. In 3 out of 12 cases on introduction of acetate there was a lowering of pressure in the aboral balloon at the time the rise began in the oral balloon.

Table 3 shows similar results with 0.2 *N* sodium butyrate at pH 7.4.

The average increase in "systolic" pressure was 14.5 cm. H_2O for the oral and 13.7 for the aboral balloon in the acetate experiment or the same within limits of measurement. Not enough experiments were made with butyrate to prove it acted differently.

TABLE 4

PER CENT CELLULOSE + HEMI-CELLULOSE	FRUIT	PER CENT CELLULOSE + HEMI-CELLULOSE	VEGETABLES
2.5	Applesauce	1.5	Asparagus
10.0	Apricots, dried and stewed	5.1	Beans (baked)
3.4	Bananas	2.5	Beets
7.3	Blackberries	91.5	Bran
1.0	Cantaloupe	4.2	Broccoli
1.5	Cherries	4.8	Brussels sprouts
4.2	Cranberries	2.5	Cabbage
8.7	Currants (black)	2.9	Carrots
4.1	Damson plums	2.4	Cauliflower
8.7	Dates	1.8	Celery
18.5	Figs, dried	0.4	Cucumber
0.4	Grapes, black	2.5	Egg plant
0.9	Grapes, white	2.2	Endive
0.6	Grapefruit	3.9	Leeks
2.0	Oranges	1.4	Lettuce
1.4	Peaches	2.5	Mushrooms
2.5	Pears	1.3	Onions
2.0	Plums	4.0	Parsnips
7.4	Raspberries	5.2	Green peas
2.2	Strawberries	2.1	Potatoes
		0.5	Pumpkin
	NUTS	1.0	Radishes
14.3	Almonds	2.6	Rhubarb
9.0	Brazil nuts	2.7	Rutabagas
6.8	Chestnuts	6.3	Spinach
13.6	Coconut	2.3	Sweet potatoes
8.1	Peanuts	1.5	Tomatoes
5.2	Walnuts	2.8	Turnips
		3.3	Water cress

Comment: As small a quantity as 1 millimol of acetate or butyrate in the duodenum caused a marked regional increase in pressure that traveled aborally as a peristaltic wave. Since many millimols of acetate and butyrate are present in the intestine at any one time this lends support to the idea that they may be factors in intestinal motility. Representative values of roughage, cellulose and hemicellulose, are given in table 4.

SUMMARY

1. After injection of 100 cc. of 0.2 *N* sodium acetate into the duodenum, absorption of acetate from the gut raised the level of volatile fatty acid in the portal vein for 120 minutes, but the liver removed most of it so that there was very little or no rise in the systemic arteries and veins.

2. Intravenous injection of isotonic sodium acetate at pH 7.4 faster than it could be metabolized stimulated the motor activity of the duodenum.

3. With only one balloon in the gut it was not always possible to observe whether acetate introduced into the stomach stimulated motor activity of the intestine, although vomiting or defecation occurred.

4. Replacement of one fourth of the Ringer-Locke fluid (in which isolated gut segments were suspended) with isotonic, isohydric, cationic-balanced acetate always resulted in marked increase in the amplitude of the rhythmic contractions of the gut. The effect was more marked in the duodenum and less in the terminal ileum and colon. In case the preparation had been in the Ringer-Locke fluid so long that the rhythmic contractions had ceased, acetate stimulated tonic contractions.

5. Acetic acid depressed the motor activity of the excised gut (H ion effect).

6. Butyrate acted like acetate and butyric acid like acetic acid.

7. Cutting the gut wall longitudinally depressed motor activity. Turning the gut inside-out abolished the motor activity as registered in a longitudinal direction. This is associated with relief of tension on the peritoneal and longitudinal muscle side. Cutting the inside-out gut wall longitudinally partially restored the activity (to the level of the normal gut that is cut). Sewing up abolished the effect of cutting.

8. Introduction of 5 cc. of acetate or butyrate through a permanent fistula in the duodenum caused increased amplitude of the rhythmic contractions recorded by a balloon just aboral to it in 15–200 seconds and of a second balloon 7 cm. aboral to the first in 40–250 seconds, indicating a wave of excitement passing down the gut. The pressure ("systolic") was raised 7–17 cm. of water and the base line ("diastolic") was raised 4–10 cm. of water.

We are indebted to Dr. J. Earl Thomas for the use of dogs with permanent gastric and duodenal fistulae and to Dr. M. J. Oppenheimer for dogs with Bickel or Thiry-Vella loops.

(1) McClendon, J. F.: J. Biol. Chem., **87:** (Proc. XXIV, vii), 1930.
(2) Grove, E. W., Olmsted, W. H., and Koenig, K.: J. Biol. Chem., **85:** 127, 1929.
(3) Olmsted, W. H., Curtis, G., and Timm, O. K.: J. Biol. Chem., **108:** 645, 1935.
(4) Williams, R. D., and Olmsted, W. H.: J. Nutrition, **11:** 433, 1936.
(5) Frazer, L. M.: Am. J. Physiol., **72:** 119, 1925.

(6) Dunham, B. S.: Am. J. Dis. Child., **29:** 200, 1925; ibid, **32:** 384, 1926.
(7) Morgan, C. J., and Thomas, J. E.: Am. J. Physiol., **97:** 546, 1931.
(8) Macfadyen, A., Nencki, M., and Sieber, N.: J. Anat. Physiol., **25:** 390, 1891.
(9) Bergeim, O., Hanszen, A. H., Pincussen, L., and Weiss, E.: J. Infect. Dis., **69:** 155, 1941.
(10) Gunn, J. A., and Underhill, S. W. F.: Quart. J. Exp. Physiol., **8:** 275, 1914.
(11) Polansky, J. B.: Am. J. Physiol., **83:** 488, 1928.
(12) McClendon, J. F.: J. Biol. Chem., **154:** 357, 1944.
(13) Barcroft, J., McAnally, R. A., and Phillipson, A. T.: Biochem. J., **38:** Proc. iv., 1944.
(14) Thomas, J. E., Crider, J. O., and Morgan, C. J.: Am. J. Physiol., **108:** 683, 1934.
(15) Thomas, J. E., and Crider, J. O.: Am. J. Physiol., **111:** 124, 1935.
(16) Alvarez, Walter C.: "An Introduction to Gastro-enterology." Paul B. Hoeber, N. Y., 1940.

J. Bacteriol. 56: 547-553, (1948)

THE INFLUENCE OF SODIUM ACETATE UPON THE DISSOCIATION OF A STRAIN OF HEMOLYTIC STREPTOCOCCUS

ALICE P. McILROY, A. E. AXELROD, AND RALPH R. MELLON[1]

Institute of Pathology of the Western Pennsylvania Hospital and the Department of Chemistry of the University of Pittsburgh, Pittsburgh, Pennsylvania

Received for publication July 24, 1948

It is generally recognized that a variety of environmental factors may influence the process of bacterial dissociation. Inasmuch as most of these studies have been conducted with media composed of crude natural materials, it was considered advisable to employ media of known chemical constitution in order to determine more precisely the nutritional factors capable of implementing this variation phenomenon. The inquiry is particularly pertinent in view of the current tendency to explain dissociative behavior, as well as other categories of variation, on a mutation basis. According to Braun (1947), the chief influence of environment is to select out the spontaneously occurring mutants.

The present paper is the first of a series dealing with the use of such "synthetic" media in studies upon bacterial dissociation. The term "synthetic" as used in this work designates media in which a hydrolyzate from purified casein is the only substance the chemical constitution of which is not absolutely established. This study is concerned with the role of sodium acetate in the maintenance of the mucoid character and virulence of a strain of hemolytic streptococcus.

METHODS

Culture. A culture of hemolytic streptococcus, Lancefield group C, Stoddard strain (Mellon and Cooper, 1938), was used throughout the investigation. This culture is mucoid in character and has a high virulence for white mice (MLD = 2 to 6 organisms). As a stock culture, it has been carried in semisolid veal heart infusion agar (0.2 per cent) containing 5 per cent defibrinated rabbit's blood. This medium is adjusted to pH 7.6. The culture has been passed through mice at frequent intervals in order to maintain its virulence.

Determination of virulence. Virulence tests were conducted by preparing varying dilutions of actively growing cultures in veal heart infusion broth and injecting 0.5-ml quantities intraperitoneally into white mice weighing 20 to 25 grams. Each dilution was administered to three mice and the animals were observed for ten days. The MLD was taken as the minimum number of organisms necessary to cause death in two of the three animals in each group.

Special media. The three synthetic media employed are shown in table 1. All experiments were conducted with 5 ml of single strength medium per culture tube.

[1] We are indebted to Lederle Laboratories, Incorporated, for the synthetic folic acid, and to Merck and Company for the pyridoxal hydrochloride and the pyridoxamine dihydrochloride.

TABLE 1

*Constituents of media**

MEDIUM A†			MEDIUM B		
Constituents	Concentration‡		Constituents	Concentration‡	
Casein hydrolyzate§	100	ml	Casein hydrolyzate§	100	ml
KCl	6	g	Sodium acetate·$3H_2O$	10	g
$Na_2HPO_4 \cdot 12H_2O$	2	g	Sodium citrate·$2H_2O$	1	g
KH_2PO_4	5	g	Glucose	40	g
$MgSO_4 \cdot 7H_2O$	1	g	K_2HPO_4	1	g
Glucose	66.5	g	KH_2PO_4	1	g
L-Cystine	270	mg	L-Cystine	200	mg
Uracil	20	mg	L-Asparagine	200	mg
Adenine	20	mg	Guanine	20	mg
Nicotinic acid	2	mg	Uracil	20	mg
Pyridoxine hydrochloride	2	mg	Adenine	20	mg
Calcium pantothenate	8	mg	Nicotinic acid	2	mg
Thiamine hydrochloride	2	mg	Pyridoxine hydrochloride	2	mg
Riboflavin	1	mg	Calcium pantothenate	8	mg
L-Tryptophan	40	mg	Thiamine hydrochloride	2	mg
$CuSO_4 \cdot 5H_2O$	2	mg	Riboflavin	1	mg
$ZnSO_4 \cdot 7H_2O$	2	mg	L-Tryptophan	40	mg
$FeSO_4 \cdot 7H_2O$	2	mg	Pyridoxal hydrochloride	2	mg
$MnCl_2 \cdot 2H_2O$	0.8	mg	Pyridoxamine dihydrochloride	2	mg
$CaCl_2 \cdot 2H_2O$	20	mg	Xanthine	20	mg
Biotin	0.0004	mg	*p*-Aminobenzoic acid	0.1	mg
Adjust to pH 6.5‖ and autoclave			*i*-Inositol	1	mg
			$MgSO_4 \cdot 7H_2O$	400	mg
			$FeSO_4 \cdot 7H_2O$	20	mg
			$MnSO_4 \cdot 2H_2O$	12	mg
			NaCl	20	mg
			$CuSO_4 \cdot 5H_2O$	2	mg
			$ZnSO_4 \cdot 7H_2O$	2	mg
			$CaCl_2 \cdot 2H_2O$	20	mg
			Folic acid	0.001	mg
			Biotin	0.0004	mg
			Adjust to pH 6.5‖ and autoclave		
$NaHCO_3$¶	4	g	$NaHCO_3$¶	4	g
L-Glutamine¶	400	mg	L-Glutamine¶	400	mg
Thioglycolic acid¶	260	mg	Thioglycolic acid¶	260	mg
Final pH	7.5		Final pH	7.5	

* Medium C was prepared by omitting the following compounds from Medium B—sodium citrate, L-asparagine, guanine, pyridoxal hydrochloride, pyridoxamine dihydrochloride, xanthine, *p*-aminobenzoic acid, *i*-inositol, and folic acid.

† Medium A is essentially that of Bernheimer *et al.* (1942).

‡ Concentration per liter of double strength medium.

§ Casein hydrolyzate, 10 per cent, "vitamin-free," acid-hydrolyzed (SMACO).

‖ The pH was adjusted with 10 per cent sodium hydroxide solution.

¶ Added aseptically to the autoclaved medium.

The glutamine solution was sterilized by filtration through a sintered glass filter. Sodium bicarbonate and thioglycolic acid were sterilized as described by Adams and Ris (1945).

Inoculation procedures. For each experiment a stock culture of maximum virulence was plated on blood agar and incubated 18 to 20 hours at 37 C. A tube of the synthetic medium (table 1) under investigation was inoculated with this culture. After incubation at 37 C for 18 hours, the cells were separated by centrifugation, washed with normal saline, and resuspended in 5 ml of saline. This suspension (0.1 ml) was used to inoculate the original synthetic medium or any modification thereof. After incubation for 4 to 5 hours at 37 C, the growth and pH measurements were made on duplicate cultures. The amount of growth was determined turbidimetrically with an Evelyn photocolorimeter using a 660 filter, and by the plate count method. The pH measurements were made with a Beckman pH meter. After storage overnight at 5 C, 0.2 ml of the culture were used to inoculate a fresh batch of the same medium. This procedure was repeated for every daily serial transfer. Virulence tests were performed on cultures from the final transfer.

In the early experiments, an 18-hour incubation period was employed in the serial transfers. The results obtained with this procedure did not differ from those obtained when the shorter incubation periods were used. The latter procedure has the advantage of avoiding effects due to the lowered pH and to the accumulation of metabolic products found in aging cultures.

EXPERIMENTAL RESULTS

In the original experiments employing synthetic medium A (table 1) a decrease in mucoid character and mouse virulence was noted after daily, serial transfers. Since this culture maintained both of these characteristics on a veal heart infusion blood medium, it is apparent that a constituent (or constituents) present in the natural medium and missing from the synthetic medium A was essential. Experiments to determine the nature of this essential factor (or factors) were initiated.

Our first efforts were directed toward an attempt to prepare a synthetic medium that was comparable to the natural medium in its ability to maintain mucoidness and virulence. It was hoped that a systematic elimination of various components of such a complete synthetic medium would lead to the identification of the essential factor(s). Accordingly, medium A was supplemented with a variety of compounds to yield medium B (table 1). This medium was capable of maintaining the mucoid character and virulence on daily, serial transfer. A systematic elimination of the various components of medium B was undertaken, and it was found that the omission of sodium acetate resulted in a medium which permitted the mucoid to smooth transformation. Sodium acetate was specific in this regard. It should be noted that the original medium employed (medium A) did not contain sodium acetate, thus explaining its inability to maintain the mucoid character.

The omission of the following compounds from medium B affected neither growth nor the ability to maintain a mucoid state: sodium citrate, L-asparagine, guanine, pyridoxal hydrochloride, pyridoxamine dihydrochloride, xanthine, *p*-aminobenzoic acid, *i*-inositol, and folic acid. To simplify our procedure,

these constituents were omitted from medium B to yield medium C (table 1). All of the subsequent results reported in this paper were performed with medium C, or with medium C lacking sodium acetate.

On the third to fourth transfer in the medium lacking acetate, transformation from the mucoid into the smooth phase became evident. At the sixth transfer only the smooth phase was present. As the cultural characteristics changed, the morphology of the organism also underwent change. The original mucoid culture composed of encapsulated cocci in very short chains dissociated to a long-chained streptococcus with loss of capsular material. Identical results were obtained in ten experiments. No evidence of any change from the mucoid phase has been noted after 64 daily, serial transfers in either medium C (containing acetate) or in veal heart infusion blood broth.

The virulence of the smooth phase for mice was considerably less than that of the mucoid phase. Thus the MLD of the mucoid phase (grown in veal heart infusion blood broth or in medium C) and the smooth phase (grown in medium C minus acetate) was 5 and 5,000 organisms, respectively.

The stability of the smooth phase was investigated. When subcultured in medium C, no transformation into the mucoid phase was noted after 14 daily, serial transfers. The decreased mouse virulence of the smooth phase was also unaffected by this procedure. In contrast, when passed serially through mice in numbers large enough to cause death, a pure culture of the smooth phase gradually reverted to the mucoid phase. At the tenth serial transfer, only the mucoid phase was evident. The virulence of this culture was identical with that of the original mucoid organism.

As determined both by the plate count and turbidimetric measurements, the omission of acetate from medium C was without effect upon the extent of growth of the mucoid phase during the first few serial transfers. After continued subculture, transformation into the smooth phase occurred. The growth rates of this resulting smooth phase in both acetate-free and acetate-containing media were identical and equal to that of the original mucoid culture. The pH of cultures grown both in the presence and absence of acetate never fell below 7.0. To further eliminate the possibility that the buffering action of acetate was significant in the maintenance of the mucoid phase, cultures were serially transferred in media in which the acetate was substituted by phosphate, citrate, and bicarbonate buffers. The mucoid phase could not be maintained in the presence of these buffers, thus demonstrating that the activity of acetate was not due to its buffering capacity.

DISCUSSION

The application of "synthetic" media to studies upon bacterial dissociation possesses a twofold advantage. First, the reproducibility of the medium can be maintained within rather narrow limits. This is of particular importance since there is formidable evidence that variations in environment can markedly influence the process of bacterial dissociation. The extreme consistency of the results reported here bears witness to the reproducibility of our environmental

bacteria. The past experience of this laboratory in studies of this type has been entirely with media composed of crude, natural materials and a not infrequent inability to reproduce certain results has been noted. It is conceivable that variations in the composition of the natural materials contained in these media could account for some of these anomalous results.

In the second place, a medium of known chemical constitution is essential in studies designed to determine the role of individual nutrilites upon bacterial dissociation. The flexibility of such media in regard to the ease with which the individual components may be varied is evident.

The present experiments have demonstrated that the elimination of sodium acetate from a "synthetic" medium determines the transformation from the mucoid to the smooth growth phase. It should be noted that as long as the mucoid phase is able to grow without acetate, the absence of the latter affects neither the growth rate of the organism nor the final pH of the culture medium. The M to S phase change, therefore, is due primarily to the absence of sodium acetate and is not the result of secondary influences upon rate of growth or degree of acidity. This point requires emphasis since changes in the pH of the medium, and bacteriostatic changes particularly, are well known to affect the incidence of bacterial dissociation. The fact that the M phase is replaced by the S phase in the absence of acetate is not interpreted as a bacteriostatic or bactericidal effect, but rather a change incident to a nutritional deficiency.

With regard to the mechanism of action of the sodium acetate, we have adopted the tentative view that this compound functions not as a growth factor but as a precursor in the biosynthesis of some higher molecular weight compound(s) which is essential – perhaps, as a structural component of the capsular material – for the maintenance of the mucoid state. Apparently an exogenous source of acetate is required for this function. In the absence of added acetate, the transformation to a smooth phase, which is stable even in the presence of acetate, occurs.

The alternate view, namely, that acetate is essential for the growth of the mucoid and not the smooth phase, is difficult to substantiate, since transformation into the smooth phase occurs following continued subculture in the absence of acetate. At least for the first few serial transfers into a medium lacking acetate (before the M to S change is recognizable) no bacteriostatic effect is noted.

The precise nature of the postulated compound into which acetate is presumed to be converted is unknown. There is ample evidence that acetate may serve in the biosynthesis of a variety of tissue constituents, such as glycogen, uric acid, fatty acids, cholesterol, dicarboxylic amino acids, and protoporphyrin (Bloch, 1947). Guirard *et al.* (1946) have reported on the probable role of acetate as a precursor of various lipoidal substrates in bacterial metabolism.

Preliminary experiments have indicated that lipids may replace acetate for the maintenance of the mucoid character of our strain of hemolytic streptococcus.

The relationship between mucoid character and virulence is not clear. In the group C organism employed in this study, maximum virulence is characteristic

only of the mucoid state. The conditioning influence of the acetate on the mucoid state in these experiments tends to emphasize the likelihood that mucoid nature and virulence do not represent linked characters, but rather an association of characters. It is interesting to note in this connection that our experience with certain strains of group A streptococci has indicated that an exogenous source of acetate is not necessary for either the growth or the maintenance of mucoid character. Although the majority of group A strains are highly virulent in the mucoid phase, it has long been known that exceptions occur. In the case of group A streptococci, it is well known that virulence is dependent upon the presence of the M antigen, and the presence of this antigen gives rise to the so-called "matt" appearance that is characteristic of the colonies produced by virulent, but nonmucoid, strains of group A streptococci.

The current genetic appraisal of this M to S transformation would probably say that we are dealing with a very low frequency of mutation rate on the part of the mucoid phase, which phase has a suppressive effect on the appearance of the resulting S mutant. This suppressive effect would be abrogated by the absence of acetate, which would select out the occasional mutant S cell by depriving the M cells of a chance to grow.

This interpretation, however, fails to accord with the results of experiments designed to test its validity. Thus two single S phase cells were mixed in acetate containing broth containing 5,000,000 M phase cells. After 18 hours' incubation at 37 C, equal numbers of M and S forms were present.

Furthermore, after 60 serial transfers of the M phase on blood agar had shown no S phase colonies, 12 isolated M colonies from a single plate were selected at random for test of the frequency of the M to S transformation. Transfers were made from each of six colonies into its own tube of acetate broth; and six colonies were likewise transferred into the same synthetic medium without acetate. After the fifth serial transfer in the latter medium, all six M phase cultures went over to the S phase, with only occasional mucoid colonies appearing. At this time all six M phase cultures in acetate broth remained mucoid, no S colonies appearing.

In other words, there is not the slightest evidence of a suppressive effect of M over S; in fact, the phenomenon of "population dynamics" appears to be in reverse. As to frequency, the transformation occurred in 100 per cent of the colonies tested. Thus, it seems permissible to infer that the burden of proof in this instance is shifted to the opponents of the theory of adaptive modification by specific environments.

SUMMARY

It has been demonstrated that the presence of sodium acetate in a synthetic medium is necessary in order to maintain the mucoid character and virulence of a strain of group C hemolytic streptococcus upon repeated daily subculture. The elimination of acetate permits the transformation from the virulent mucoid phase to a relatively avirulent smooth phase. The smooth phase does not revert to the mucoid state *in vitro* in the presence of acetate, but does completely

were after ten passages in mice. This phase change has been shown to be due primarily to the absence of sodium acetate and is not the result of secondary influences upon rate of growth or degree of acidity.

All the evidence to date points to the likelihood that the transformation is not the result of a spontaneous mutation, but is an adaptive response to the absence of a nutrilite so vital to the mucoid phase that this phase may be said to have no existence in its absence.

REFERENCES

Adams, M. H., and Roe, A. S. 1945 A partially defined medium for the cultivation of pneumococcus. J. Bact., **49**, 191–199.

Bernheimer, A. W., Gillman, W., Hottle, G. A., and Pappenheimer, A. M., Jr. 1942 An improved medium for the cultivation of hemolytic streptococcus. J. Bact., **43**, 495–498.

Bloch, K. 1947 The metabolism of acetic acid in animal tissues. Physiol. Revs., **27**, 574–620.

Braun, Werner 1947 Bacterial dissociation. Bact. Revs., **11**, 75–114.

Gibbard, B. M., Snell, E. E., and Williams, R. J. 1946 The nutritional role of acetate for lactic acid bacteria. Arch. Biochem., **9**, 361–379, 381–386.

Mellon, R. R., and Cooper, F. 1938 Biphasic nature of certain alpha-prime hemolytic streptococci. Proc. Soc. Exptl. Biol. Med., **38**, 158–160.

Fatty Acid Metabolism

Cancer Research 16(1): 57-62 - (1956)

VIII. Acetate Metabolism *in Vitro* during Hepatocarcinogenesis by *p*-Dimethylaminoazobenzene*

GRACE MEDES, BERNICE FRIEDMANN, AND SIDNEY WEINHOUSE

(Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia, Pennsylvania)

Previous studies have revealed that transplanted tumors, including the hepatoma, display a distinctive pattern of fatty acid metabolism which bears little resemblance to that of liver, being more characteristic of nonhepatic tissue. Thus, tumor slices oxidize fatty acids but produce little or no ketone bodies (19); they utilize acetoacetate more readily than do liver slices (19); and they synthesize relatively little fatty acid from acetate as compared with liver slices (7, 8). With regard to the metabolism of glucose, Olson (11) has found that the carcinogen-induced primary liver tumor also differs markedly from its tissue of origin, since it oxidizes glucose about 10 times more rapidly, does not store glycogen appreciably, and synthesizes fatty acid from glucose much more rapidly than does liver. As an extension of our previous studies in transplanted tumors, the present work was undertaken to learn whether the pattern of metabolism of fatty acids in a primary liver tumor also differs from its tissue of origin as does the transplanted hepatoma, and, if so, whether such metabolic changes appear during the "preneoplastic" period.

Many differences in activities of individual enzymes between the normal and "preneoplastic" liver, and between these and the primary liver neoplasm, have been reported (3); as yet, however, few data are available concerning the metabolism of foodstuffs by these tissues. The possibility that changes in fatty acid metabolism in the preneoplastic livers of carcinogen-fed rats might precede the formation of the tumor can be anticipated from the marked changes which have been reported in mitochondrial content (13-15), in the levels of coenzymes (17), and in enzymatic activities associated with protein synthesis (15).

Because of its central position in metabolism, acetic acid was selected as a substrate for this study. Acetic acid in the form of its coenzyme-A ester is established as a common intermediate in the metabolism of both glucose and fatty acids and is also an immediate precursor of the carbon skeletons of fatty acids, cholesterol, and ketone bodies. These experiments were conducted according to the following plan. Rats on an 18 per cent casein, low-riboflavin diet containing 0.06 per cent *p*-dimethylaminoazobenzene (DAB), were sacrificed at approximately semimonthly intervals during the course of about 400 days. The livers were sliced and incubated in Warburg vessels with acetate-2-C^{14}. Where tumors developed, these were studied in the same manner. Control animals, on the same diet without DAB, were studied concomitantly. The following metabolic transformations of acetate were studied: its oxidation to CO_2 and its conversions to acetoacetic acid, to fatty acids, and to cholesterol. A similar investigation of glucose metabolism is being conducted, and the results will be published separately.

MATERIALS AND METHODS

p-Dimethylaminoazobenzene was selected as the carcinogen in this study, because its mild carcinogenic action and low toxicity should result in a minimum of other pathological changes and should permit a long induction period for observations of preneoplastic metabolic changes. The rats were 37- to 56-day-old males from our stock colony Carworth Farms strain. The carcinogen was present in the amount of 0.06 per cent in a diet of the following composition: General Biochemicals vitamin-free casein, 18 per cent; Crisco, 9 per cent; cod-liver oil (one-fourth of which was a halibut-liver concentrate), 1 per cent; cystine, 0.2 per cent;

* Aided by grants from the National Cancer Institute of the National Institutes of Health; the American Cancer Society recommended by the Committee on Growth of the National Research Council; and the United States Atomic Energy Commission, Contract No. AT(30-1)777. The aid of the Ruth Estrin Goldberg Memorial is also acknowledged with thanks. Technical assistance was rendered by Mr. George Paden.

Received for publication July 2, 1955.

choline, 0.2 per cent; General Biochemicals salt mixture No. 2, 4 per cent; Alphacel, 4 per cent; and a mixture of 20 parts glucose and 80 parts cane-sugar, 63.6 per cent. All the known B vitamins were added in adequate amounts except riboflavin, which was present at the level of 1 mg/kg of total diet. A control group was maintained on the same diet without the carcinogen. To make certain that the diet was nutritionally adequate for this study, another group of control rats was maintained on Allied Mills Rat Checkers throughout the experimental period, and experiments with these animals were interspersed with those on the casein diet. Rats of both control groups gained about 140 gm. of weight during the experimental period of 400 days. Those given DAB weighed 16–20 per cent less, but all animals remained ostensibly in good health, and none died during the experimental period. The first tumor appeared after 211 days of DAB-feeding.

Metabolic experiments.—From the time rats were placed on the diets, experiments were run periodically, usually at intervals of a month in the early stages, and oftener thereafter. On each occasion, experiments with a control and a DAB-fed animal were run simultaneously. The animals were prepared for the metabolic experiments by being fasted for 24–48 hours and refed a high-carbohydrate diet (6) for 24 hours. This procedure ensured adequate nutrition of the animal and thus optimal conditions for fatty acid synthesis. During the period when tumors were expected, the rats were examined regularly, and, when a tumor was evident on abdominal palpation, the animal was used promptly for an experiment.

The rats were decapitated, and samples of liver or tumor were removed for glycogen determination and histologic examination.[1] In contrast with the diffuse, multifocal tumors usually obtained on a low-protein diet, many of the tumors obtained on the 18 per cent casein diet were large, discrete, and relatively few in number in each liver. In these instances it was possible to dissect out sufficient "pure" tumor tissue for metabolic studies without including appreciable quantities of necrotic material or nontumorous liver tissue. Five gm. were then sliced with the Stadie slicer (16) and were placed in a large Warburg flask. In a few instances in which this amount of liver or tumor was not available, the amounts of saline medium and substrate were reduced in proportion, and the resulting analytical and metabolic data were corrected to correspond to values for 5 gm. of tissue.

[1] The authors are indebted to Dr. A. J. Dornfeld and the Department of Pathology of this Institute for preparation and examination of histological material.

The methyl-labeled sodium acetate was obtained from Tracerlab, Inc., on allocation by the Atomic Energy Commission. It was diluted with inactive acetate to a specific activity of between 28,000 and 35,000 counts/min. and made up in 0.4 M concentration. One ml. of this preparation was added to 39 ml. of buffer, giving a final concentration of 0.01 M. Specific activities and conversions were calculated by our standard procedures described previously (7). After incubation and collection of the respiratory CO_2, the slices and medium were transferred to large centrifuge tubes, in which the slices were separated from the supernatant fluid and washed 3 times with water. The medium and washings were combined and immediately frozen until acetoacetate could be determined (18). The washed tissues were then treated for recovery of fatty acids and cholesterol. Extraction of these substances and procedures for their isolation and purification were given in a previous publication (7).

RESULTS

Analytical and metabolic data are reported for seventeen control rats on the 18 per cent casein diet, 24 control rats on the Checkers diet, nineteen DAB-fed rats in which no tumors had yet appeared, and six rats which developed tumors in sufficient amount for metabolic studies.

Analytical data.—As shown in Table 1, livers of control rats on the 18 per cent casein diet (Group 1) had essentially the same content of glycogen, fatty acids, and cholesterol as did those on the Checkers diet (Group 6). These values are in close agreement with those reported previously for normal, nourished rats (6). To discover whether nontumor pathology has any bearing on the behavior of the preneoplastic liver,[2] the nontumorous livers of the DAB-fed rats were divided into two groups. The first, Group 2 in the table, consisted of eleven livers which showed no gross or microscopic abnormalities, and is designated as "normal." The second group of livers, Group 3, was from eight rats generally on the diet a long time; these displayed various degrees of pathological change, *viz.*, fibrosis, slight to severe cirrhosis, and irregularities in cell size and shape. Neither of these groups deviated significantly from the normal in glycogen, fatty acid, or cholesterol content.

Of the tumors obtained on DAB-feeding, some did not yield sufficient tissue for the metabolic experiments, and some were used for other studies. In all, six were used in the present investigation:

[2] The term "preneoplastic" is used merely to indicate the liver of the DAB-fed rat. It does not connote any gross or microscopic pathology.

two were classified histologically as cholangiomas and four as hepatocarcinomas. Their content of cholesterol, fatty acids, and glycogen (Group 5) may be compared with the adjacent nontumorous liver lobes, Group 4. All the livers in Group 4 were moderately to severely cirrhotic; nevertheless, the levels of these components were essentially those of normal liver. The glycogen values were somewhat below normal; however, if two livers, containing only 25 and 45 mg. are omitted, the average would have been 301 mg/gm, which is not far from the normal level. Since these animals, in which large tumors are present, do not eat with the appetite of normal animals, the somewhat lower glycogen levels can probably be attributed to this factor rather than to a loss in the ability to store glycogen. In contrast, it appears that the liver tumor has largely lost this capacity. In none was the glycogen content higher than 20 mg/5 gm, and the average was only 11 mg. The cholesterol and fatty acid levels were a little higher than, but in the same range as, their levels in liver.

Metabolic data.[3]—In Table 2 there are presented data on the various metabolic transformations of acetate-2-C^{14} in the tissues under study. In this table the groups are the same ones listed in

[3] Originally, a series of experiments was performed on a group of rats maintained on a 12 per cent casein diet with 0.06 per cent DAB, on which Kline *et al.* (5) obtained a tumor incidence of 100 per cent after 4 months. These were abandoned, however, when it was found that livers of control rats on this diet, owing to a deficiency of protein or lipotropic factors or both, displayed a marked impairment in lipogenesis. However, the impairment was no greater in the precoplastic livers of the DAB-fed than in the control animals, and except for a relatively high fat content these livers were not singularly different in other metabolic behavior from those of animals on the 18 per cent casein diet.

TABLE 1

ANALYTICAL DATA FROM LIVERS AND TUMORS OF RATS DURING CARCINOGENESIS WITH *p*-DIMETHYLAMINOAZOBENZENE

All values are in mg/5 gm fresh weight of tissue. Ranges are given in parentheses.

Group no.	DAB	No. rats	Days on diet	Condition	Glycogen (mg.)	Fatty acid (mg.)	Cholesterol (mg.)
1	−	17	53-456	Normal	405 (265-545)	102 (83-131)	7.4 (6.1-9.0)
2	+	11	53-203	Normal	390 (260-510)	99 (84-149)	7.3 (5.1-9.9)
3	+	8	133-456	Pathological No tumors	360 (76-550)	96 (75-127)	10.4 (6.5-16.0)
4	+	6	211-367	Nontumor lobes of tumorous liver	216 (25-480)	90 (82-107)	10.4 (7.8-13.0)
5	+	6	211-367	Tumors	11 (2.5-20)	127 (79-208)	15.0 (13.2-16.8)
6	−*	21	32-363	Normal	450 (250-685)	99 (63-165)	7.0 (4.5-9.1)

* Allied Mills Rat Checkers diet.

TABLE 2

CONVERSIONS OF ACETATE-2-C^{14} IN LIVERS OF RATS DURING CARCINOGENESIS WITH *p*-DIMETHYLAMINOAZOBENZENE

Tissue slices were incubated with labeled acetate for 4 hours at 37.6° C. with oxygen in the gas phase. Values are calculated on the basis of 5 gm. fresh weight of tissue.

Group no.	DAB	No. rats	Oxygen consumption (μM)	$BaCO_3$ Amount (μM)	$BaCO_3$ R.S.A. (Per cent)	$BaCO_3$ Acetate incorporated (μatoms C)	Acetoacetic acid Amount (μM)	Acetoacetic acid R.S.A. (Per cent)	Acetoacetic acid Acetate incorporated (μatoms C)	Fatty acid Acetate incorporated (μatoms C)	Cholesterol Acetate incorporated (μatoms C)
1	−	17	1130 (830-1390)	1150 (820-1450)	6.24 (4.87-8.35)	70.4 (42.5-102.4)	129 (66-261)	26.5 (16-44)	180 (56-212)	19.8 (10.8-25.0)	1.40 (0.52-2.60)
2	+	11	1010 (850-1210)	1050 (850-1190)	5.08 (3.48-6.13)	55.2 (31.6-96.8)	83 (52-99)	27.3 (21-38)	90.0 (60-204)	21.0 (8.4-36.8)	1.84 (0.76-8.16)
3	+	8	570 (640-1570)	1000 (670-1790)	5.90 (3.26-6.17)	57.2 (19.2-84.0)	68 (22-194)	29 (11-41)	63.2 (40-156)	20.4 (0.12-41.8)	3.04 (0.41-6.12)
4	+	6	1370 (1140-1470)	1310 (1110-1590)	7.40 (6.75-9.29)	96.2 (77.2-115.6)	76 (55-125)	29 (28-33)	87.0 (64-156)	24.0 (8.4-44)	2.05 (0.52-3.60)
5	+	6	600 (690-1130)	850 (670-1090)	6.37 (4.76-8.28)	54.8 (39.0-70.4)	22 (0-50)	14 (9-41)	13.7 (0-26.8)	2.52 (1.26-3.60)	1.04 (0.60-3.84)
6	−	21	1060 (710-1350)	1020 (740-1460)	6.48 (4.61-10.45)	70.4 (41.6-112.4)	54 (25-137)	31.1 (10-45)	70.4 (32-140)	17.9 (2.36-54.0)	1.52 (0.60-2.88)

Table 1. Both control groups showed essentially the same metabolic behavior. During the 4-hour incubation period, about 1,000 μM of oxygen were consumed, and approximately the same quantities of CO_2 were produced. The relative specific activities of the carbon dioxide indicate that less than 7 per cent of the total represents carbon from the substrate; the major portion must have been derived from endogenous metabolites of the tissue. Despite a considerable range in the total quantity of substrate carbon converted to CO_2, the average of each control group was exactly the same, 70.4 μatoms. This represents the complete oxidation of approximately 15 per cent of the substrate. Yields of acetoacetate as well as its relative specific activity varied widely in the different livers. On the average, about one-half as much was produced in livers of rats on the Checker diet as from those on the casein diet. An observation of interest was the high relative specific activity of the acetoacetate, which, on the average, was about 30 per cent, or more than 4 times that of the CO_2.

Incorporation of acetate carbon into the lipids followed the pattern previously observed (6). A wide range of incorporation was displayed but was generally high in the control livers; and the incorporation of acetate into fatty acids was about 10 times that into cholesterol.

Comparison of Groups 2 and 3 with 1 and 6 makes it evident that the feeding of DAB over long periods does not affect the utilization by liver of acetate for oxidation to CO_2 or for conversion to acetoacetate, fatty acids, or cholesterol. Even the liver tissue of Group 4, adjacent to well developed tumors, did not differ appreciably from the control livers in these respects. In contrast, the tumors (Group 5), though displaying almost unimpaired capacity for oxidation of acetate to CO_2 or for conversion to cholesterol, had a markedly lower capacity for ketogenesis and for fatty acid formation from acetate.

The average of 990 μM oxygen consumed by the tumor slices, as well as the range of variation of from 690 to 1130 μM, was well within the range exhibited by the normal liver slices. The average CO_2 production of 54.8 μM in the tumor was somewhat lower than that of 70.4 in liver slices, but the ranges overlapped considerably. The values for cholesterol formation were likewise within the ranges of both the normal and the preneoplastic livers. However, incorporation of acetate into acetoacetate in the tumors was far below that in liver. The average incorporation was only 13.7 microatoms, which was one-sixth of the average for the adjacent liver tissues; and the highest value for a tumor, 26.8 μatoms, was less than any value given by liver. Incorporation of acetate into fatty acids in the tumor slices was on the average less than one-eighth that of liver slices, and again was considerably below the lowest value found for adjacent liver tissue.

DISCUSSION

The persistence of certain biochemical features of differentiated tissue throughout the neoplastic transformation has been well documented (3, p. 358). As examples, one may cite the production of melanin in melanomas, bone formation in osteogenic sarcomas, and hormone secretion in certain glandular tumors. It is thus of unusual interest that such specialized functions of the normal liver cell as the accumulation of glycogen and the formation of acetoacetate and fatty acids are largely lost in the primary liver tumor.

The abrupt change in the metabolic pattern on the advent of the tumor is clearly displayed in Chart 1, which also shows graphically how glycogen content, ketogenesis, and lipogenesis remain essentially constant throughout the period of tumor induction. The glycogen levels in livers of the DAB-fed rats are as high, throughout the whole induction period of over a year, as in the controls, and this was, on the whole, true for the incorporation of acetate into acetoacetate and fatty acids. Only in the tumor is there almost complete failure in glycogenesis, ketogenesis, and lipogenesis.

No trend was apparent which would indicate that these changes, observable in neoplastic cells, had occurred generally throughout the mass of liver cells prior to the appearance of the tumor. The possibility remains, of course, that metabolic changes may have taken place in a relatively small number of cells destined ultimately to become neoplastic; if so, their number was too small to affect the essentially normal pattern of fatty acid metabolism of the preneoplastic liver.

Other investigators have also observed abrupt changes in metabolism on initiation of primary, induced cancer, with little or no prior deviations during the preneoplastic period. Hayashi and Tomita (4) observed that liver tumors in rats, caused by feeding of o-aminoazotoluene, displayed a marked increase in glycolysis, and similar observations were made during liver tumorigenesis with DAB by Nakatani *et al.* (10). The latter investigators, however, also observed an increase in anaerobic glycolysis in the preneoplastic liver, and essentially similar observations were made by Berk *et al.* (1). In a subsequent study, Orr and Stickland (12) found that glycolysis in liver is in reality glycogenolysis and is variable in normal liver, being dependent on the glycogen content. They found that

the ability to glycolyze exogenous glucose was very low in liver, whether normal or preneoplastic up to incipient tumor formation. This characteristic property of tumor cells appeared only with onset of tumor formation. In confirmation of these results, Dickens and Weil-Malherbe (2) also observed a high aerobic and anaerobic glycolysis in the DAB-induced hepatoma, with little or no change from normal in the adjacent nontumorous portions of liver tissue. Among other changes, they observed in the tumor marked decreases in such specialized liver functions as urea, acetoacetate, and sugar synthesis, and in the oxidation of uric acid. These changes were unaccompanied by appreciable deviations from normal in the surrounding liver tissues.

From previous studies on tumor induction by carcinogens it is evident that the cellular transformation leading to carcinogenesis occurs early. The Millers (9) have shown that maximum dye binding to liver proteins occurs at 4–5 weeks following introduction of DAB into the diet. It is well established that feeding of certain azo dyes for periods of about 3–4 months results inevitably in 100 per cent tumor induction whether or not carcinogen administration is continued thereafter. Thus, an irreversible change in the liver cell must have occurred by this time, which leads inevitably to a

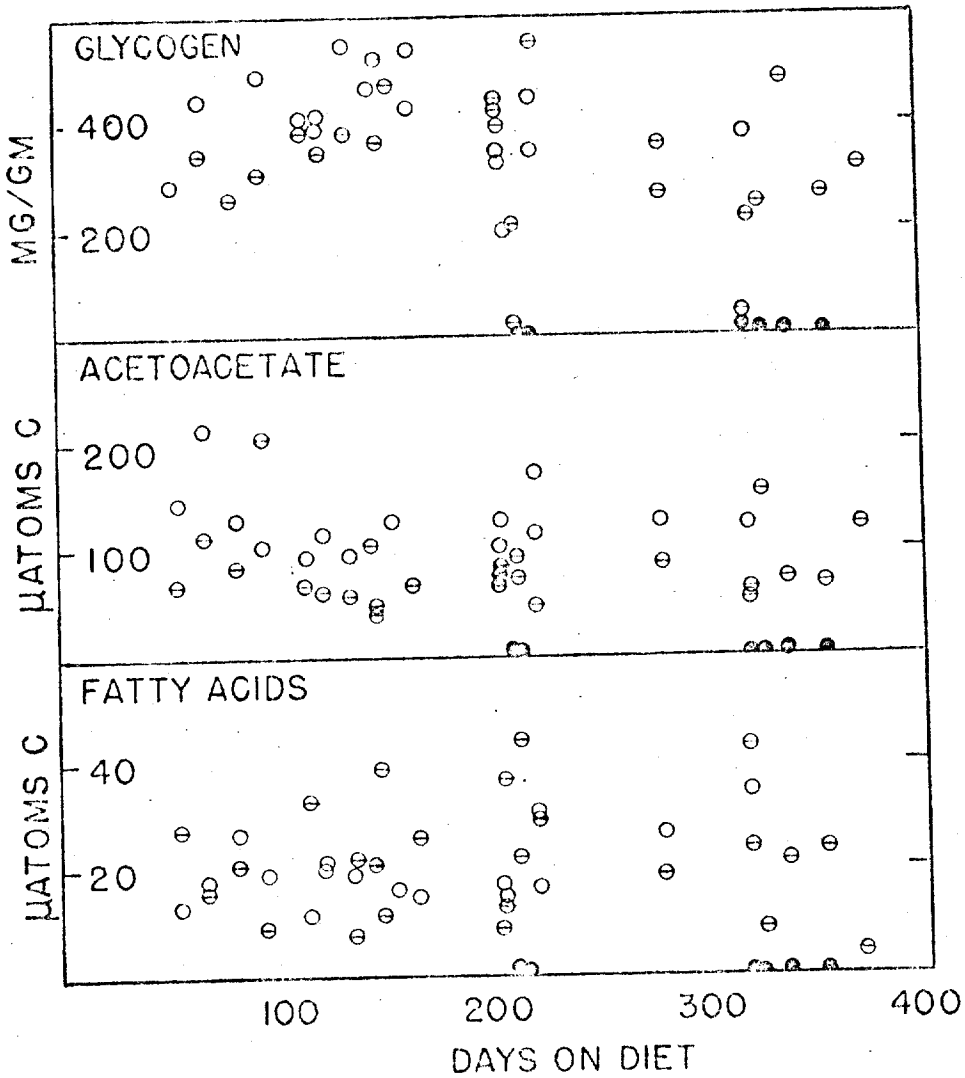


CHART 1. Glycogen content and acetoacetate and fatty acid formation in livers of normal and DAB-fed rats during carcinogenesis, and in tumors so derived. Open circles represent the control livers; lined circles, the DAB-fed rat livers; and solid circles, the tumors.

neoplasm. Whatever may be the ultimate relationship of this change to the carcinogenic process, and thus far no such relationship is evident, apparently it does not involve and is not associated with deviations in the normal hepatic pattern of fatty acid metabolism.

SUMMARY

The following conversions of acetate-2-C^{14} were studied in normal and preneoplastic liver slices of rats fed *p*-dimethylaminoazobenzene and in tumors arising thereby: oxidation to CO_2 and formation of acetoacetate, fatty acids, and cholesterol.

Conversion to CO_2 and cholesterol were essentially similar in all three tissue types, whereas acetoacetate and fatty acid formation were greatly decreased in the primary tumor as compared with either normal or preneoplastic liver. Glycogen content was also extremely low in the tumor, whereas it was essentially normal in preneoplastic liver.

Thus, it appears that the deviations in pattern of acetate metabolism displayed by the primary tumor from that of the normal liver occur abruptly with the onset of tumor formation and are not evident in the bulk of the preneoplastic liver cells.

REFERENCES

1. Burk, D.; Berkens, O. K.; and Sugiura, K. Metabolism of Butter Yellow Rat Liver Cancers. Cancer Research, 1:733-34, 1941.
2. Dickens, F., and Weil-Malherbe, H. The Metabolism of Normal and Neoplastic Tissue. XX. A Comparison of the Metabolism of Tumors of Liver and Skin with That of the Tissue of Origin. Cancer Research, 3:73, 1943.
3. Greenstein, J. P. Biochemistry of Cancer. 2d ed. New York: Academic Press, Inc., N.Y. 1954.
4. Hayashi, I., and Tosita, T. Studien über allgemeine Disposition bei experimenteller Krebserzeugung. III. Über die Entstehung von Hepatom und die Umstimmung des Gewebestoffwechsels. Gann, 31:232-38, 1937.
5. Kline, B. E.; Miller, J. A.; Rusch, H. P.; and Baumann, C. A. Certain Effects of Dietary Fats on the Production of Liver Tumors in Rats Fed *p*-Dimethylaminoazobenzene. Cancer Research, 6:5-7, 1946.
6. Medes, G.; Spirtes, M. A.; and Weinhouse, S. The Estimation of Fatty Acid Synthesis in Rat Liver Slices. J. Biol. Chem., 205:401-8, 1953.
7. Medes, G.; Thomas, A.; and Weinhouse, S. Nutritional Factors in Fatty Acid Synthesis by Tissue Slices *in Vitro*. J. Biol. Chem., 197:181-91, 1952.
8. ———. Metabolism of Neoplastic Tissue. IV. A Study of Lipid Synthesis in Neoplastic Tissue Slices *in Vitro*. Cancer Research, 13:27-29, 1953.
9. Miller, E. C., and Miller, J. A. The Presence and Significance of Bound Aminoazo Dyes in the Livers of Rats Fed *p*-Dimethylaminoazobenzene. Cancer Research, 7:468-80, 1948.
10. Nakayani, M.; Nakano, K.; and Chaiga, Y. Untersuchung über den Gewebestoffwechsel beim Verlauf der Leberkrebsentstehung durch Dimethylaminoazobenzolfütterung. Gann, 32:240-44, 1958.
11. Olson, R. E. Oxidation of C^{14}-Labeled Carbohydrate Intermediates in Tumor and Normal Tissue. Cancer Research, 11:571-84, 1951.
12. Ohe, J. W., and Stickland, L. H. The Metabolism of Rat Liver during Carcinogenesis by Butter Yellow. Biochem. J., 35:479-87, 1941.
13. Price, J. M.; Miller, J. A.; and Miller, E. C. Studies on the Intracellular Composition of Liver and Liver Tumors from Rats Fed 4-Dimethylaminoazobenzene. Cancer Research, 9:96-102, 1949.
14. Rutman, R. J.; Cantarow, A.; and Paschkis, K. E. Studies in 2-Acetylaminofluorene Carcinogenesis. I. The Intracellular Distribution of Nucleic Acids and Protein in Rat Liver. Cancer Research, 14:111-14, 1954.
15. ———. Studies in 2-Acetylaminofluorene Carcinogenesis. II. The *in Vitro* Uptake of Alanine-1-C^{14} by Preneoplastic Liver and Hepatoma Mitochondrial Protein. *Ibid.*, pp. 115-18.
16. Stadie, W. C., and Riggs, B. C. Microtome for the Preparation of Tissue Slices for Metabolic Studies of Surviving Tissues *in Vitro*. J. Biol. Chem., 154:687-90, 1944.
17. Stheemen, M. J.; Shelton, E.; and Schneider, W. C. Quantitative Morphological Studies on the Livers and Liver Homogenates of Rats Fed 2-Methyl- or 3′-Methyl-4-Dimethylaminoazobenzene. Cancer Research, 13:279, 1953.
18. Van Slyke, D. D. The Determination of Acetone Bodies in Blood and Urine. J. Biol. Chem., 83:115-28, 1929.
19. Weinhouse, S.; Allen, A.; and Millington, R. H. Metabolism of Neoplastic Tissue. V. Fatty Acid Oxidation in Slices of Transplanted Tumors. Cancer Research, 13:367-71, 1953.

Metabolic Studies with Acetic, Lactic and Citric Acids

II. *Distribution of Exogenous Acetic and Citric Acid in the Animal Body

Fedor Medzihradsky and Walther Lamprecht (with experimental collaboration of Maria Rehling)

(From the Biochemical Laboratory of Munich Technical Institute)

Source: Zeitschr für Lebensmittel-Untersuchung und Forschung 131 (4): 221-25, 1966.

The present study was carried out within the framework of investigations on acetic, lactic and citric acids in order to clarify the metabolic fate of these acids supplied to the organism. The effect of these nutritional (alimentary) acids on metabolic concentrations of intermediate products in the animal body, their excretion rates (levels) and their gluconeogenetic properties have been reported in a previous study (1). The results of these tests have shown, among other things, that, after oral administration of C^{14}-labeled acetic or citric acid, high levels of $C^{14}O_2$ are observed after a short time in the air exhaled by animals. For citric and acetic acids, the maximum $C^{14}O_2$ levels in the exhaled air are observed during the 3rd and 5th hour, resp., after per os administration and are equal to 20 and 15%, resp., of the supplied activity.

In the present studies, the time distribution of acetic and citric acids in blood, liver and kidneys is measured after peroral and intraperitoneal administration (Note: We wish to thank Dr. F. Martens for his stimulating discussion of this subject).

---

*I. cf. reference (1)

Metabolic Studies with Acetic, Lactic and Citric Acids.

II. *Distribution of Exoqenous Acetic and Citric Acid in the Animal Body

Fedor Medzihradsky and Walther Lamprecht (with experimental collaboration of Maria Rehling)

(From the Biochemical Laboratory of Munich Technical Institute)

Source: Zeitschr für LeBens mittel-Untersuchung and Forschung 131 (4): 221-25, 1966.

The present study was carried out within the framework of investigations on acetic, lactic and citric acids in order to clarify the metabolic fate of these acids supplied to the organism. The effect of these nutritional (alimentary) acids on metabolic concentrations of intermediate products in the animal body, their excretion rates (levels) and their gluconeogenetic properties have been reported in a previous study (1). The results of these tests have shown, among other things, that, after oral administration of $C^{14}O_2$ are observed after a short time in the air exhaled by animals. For citric and acetic acids, the maximum $C^{14}O_2$ levels in the exhaled air are observed during the 3rd and 5th hour, resp., after per os administration and are equal to 20 and 15%, resp., of the supplied activity.

In the present studies, the time distribution of acetic and citric acids in blood, liver and kidneys is measured after peroral and intraperitoneal administration (Note: We wish to thank Dr. F. Martens for his stimulating discussion of this subject).

---

*I. cf. reference (1)

Experimental Part

1. Materials

Female pure-blood rats weighing about 200 g were used in all tests. The animals were kept on a standard "Altromin R" feed diet, and were deprived of food 6 hours before the start of the tests. The pigeon liver extract required for the acetate determination was kindly supplied to us by Prof. Dr. O. Wieland (Note: We wish to thank Dr. D. Hepp for his friendly collaboration and suggestions for carrying out the enzymatic-colorimetric acetate determination). Coenzyme A and ATP are commercial preparations obtained from the Boehringer and Söhne firm, and the other chemicals used had a pro anal. degree of purity (pure for analysis).

2. Collection and Processing of Samples

The acids were administered in separate test series by means of a stomach probe (300 mg acetic and citric acid in 1 ml of a solution adjusted to pH 4 with NaOH) and intraperitoneally (125 mg of the Na Salts). Blood samples were taken at definite time intervals (see Fig. 1) by heart puncture on the live animal (Note: The heart punctures were carried out in collaboration with P. Balde (Cand. med.), to whom we wish to express our special thanks). The samples were deproteinated in the cold (2-4°C) with a 6% $HClO_4$ solution, centrifuged for 10 min at 1000 g, decanted, the residue was collected with perchloric acid, and the samples again centrifuged. The combined supernatants were neutralized with K_2CO_3 to pH 7 and allowed to stand 30 min at 0°C. The solutions, after being decanted from the $KClO_4$ precipitate, were used for the determination of the metabolite concentrations.

Organ samples (liver and kidneys), after decapitation of the animals, were placed quickly (within 1 min) into liquid nitrogen, then finely ground

in a mortar, and weighed in the cold (2-4°C) in perchloric acid. Further treatment was carried out as described above.

The excretion of acetate in urine was studied in separate tests after appropriate peroral administration of acetic acid through a stomach probe; the animals were then left in the metabolism cage for 72 hours, receiving only drinking water, and urine samples were collected during this time. These samples were deproteinated with perchloric acid in a 1:4 ratio, centrifuged, and substrate analyses were carried out in the neutralized supernatant.

3. Acetate and Citrate Determinations

Acetate was determined by the Lundquist method (2). In this process the biological perchloric acid extracts are subjected to a microdiffusion according to Conway, whereby acetic acid is transferred into caustic soda. The determination proper was done by enzymatic-optical means with the aid of a pigeon liver extract, which, with coenzyme A and ATP, converts acetate into acetyl-CoA, followed by acetylation of sulfanilamide. The amount of unreacted sulfanilamide is determined colorimetrically (3). The amount of converted reagent is proportional to the acetate concentration.

In view of the fact that the enzymatic acetate determination is an extremely time-consuming and difficult procedure, we have developed a method for the gas-chromatographic determination of acetic acid in blood and organs, which can be carried out in a routine-like fashion (Medzihradsky and Lamprecht (4)).

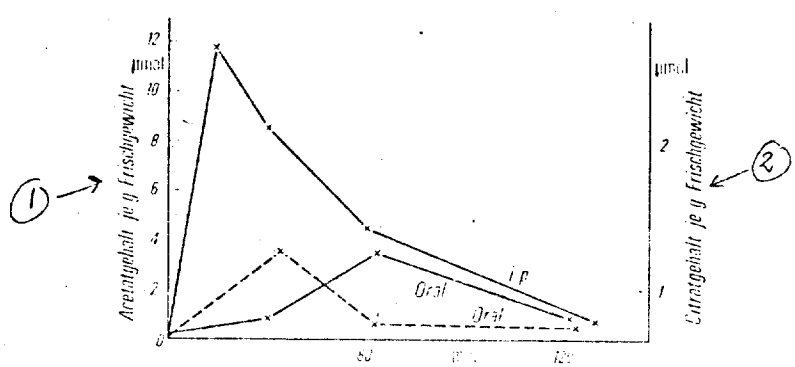
Citric acid was determined colorimetrically according to Natelson *et al.* and Elliot (5,6). In this method, citric acid is converted into pentabromoacetone with $KMnO_4$ and KBr. This compound yields with thiourea a complex, the color intensity of which is determined at 430 nm.

Results and Discussion

The results shown in the figures are average values obtained in 2-4 tests.

As can be seen from Fig. 1, both oral and intraportal administration of acetate causes only a short-lasting rise (increase) of the blood acetate content. Acetate supplied intraperitoneally as a depot substance to the animal body can already be detected in the blood after 15 min in maximum values of 12 micromoles/g fresh weight. On oral application, as a result of an intermediate resorption, maximum values (3.5 micromoles/g fresh weight) are obtained only 60 min after administration. In spite of these differences (both in regard to time and amount) in the acetate content after oral (300 mg) and intraperitoneal (125 mg) administration, the decay periods of this compound in blood are strikingly similar in both cases; 2 hours after administration, the blood acetate rise caused by the test has dropped again. The sudden rise of the blood acetate values taking place after the high intraperitoneal administration also drops within 1 hour to the maximum value achieved after oral administration. From this moment on, acetate leaves (or is eliminated from) the blood circulation system at comparable rates.

Fig. 1. Acetate and citrate content of blood after oral and intraperitoneal administration. Acetate x———x; citrate x- - - - -x.



1 - Acetate content per g fresh weight
2 - Citrate content per g fresh weight

The citrate content of blood (Fig 1) shows a similar time course as that of acetate. In agreement with earlier results (1), citric acid is swept into (or enters) the blood stream through the gastrointestinal tract at a faster rate than acetic acid, and is also eliminated earlier from the blood. A maximum content of citrate (0.9 micromoles/g fresh weight) is achieved already 30 min after administration. After 60 min, this increased citrate content again returns to normal values.

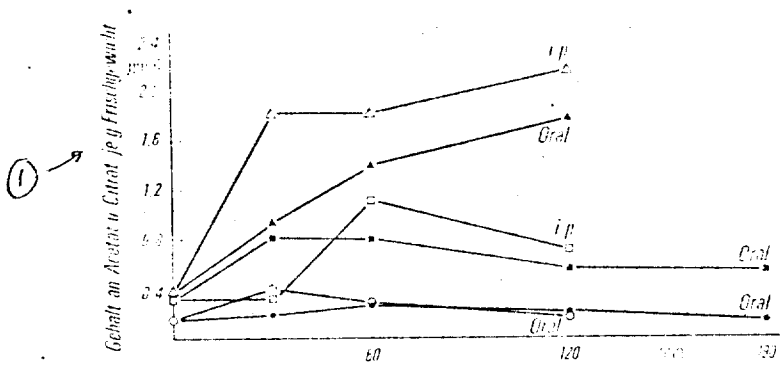
Normal values in blood amount to 0.311 micromoles acetate and 0.034 micromoles citrate per g fresh weight. In the liver and kidneys, per g fresh weight, 0.311 and 0.368 micromoles acetate and 0.147 and 0.179 micromoles citrate, respectively, are found.

The variation of the acetate content of the liver after administration corresponds to the course of the blood acetate values (Fig. 2); 60 minutes after administration, the amount of acetate converted by the liver is greater than the amount absorbed. At the same time, the acetate content of this organ does not exhibit at any time such a comparable and definite increase as in the blood. The maximum value is observed after intraperitoneal administration and is equal to approximately 3 times the normal value. Apparently, after acetate has remained in the blood for a short time, as was determined in these tests, this substrate, following absorption in the liver, is converted by this organ at a high rate. This finding corresponds to the test results obtained by Hochheuser, Weiss and Wieland (7), showing the absorption of large amounts of acetate by the isolated rat liver during perfusion.

On the other hand, Forsander, Räihä and Suomalainen (8) obtained only low $C^{14}O_2$ rates (levels) during the flow of C^{14}-ethanol through the liver. However, the $C^{14}O_2$ yield was considerably higher when blood was allowed to flow through the rear limbs of the animals. Lundquist (9), on the basis of results of his own studies, reached the conclusion that acetate is primarily and preferentially converted by peripheral organs. The explanation of this primary (preferential) position of acetate, according to which, due to its activation by acetate thiokinase, a large portion of coenzyme A made available to the cell is taken away (withdrawn) from other enzyme systems, can also be used to clarify a further experimental observation. The lack of appetite (feeling of satiety) (10), observed in animal tests after intravenous administration of acetate, could be caused by an inhibition of fatty acid degradation due to a lack of CoA.

In other studies with C^{14} labeled compounds (1), we were able to establish that when acetic and citric acids are supplied from the outside to the animal body, these acids can be utilized in a gluconeogenetic way beyound (or on top of) the reaction consequences of the citric acid cycle (?).

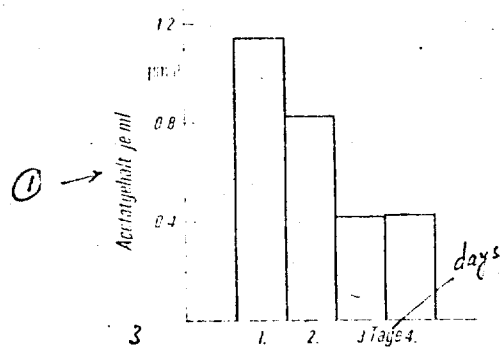
Fig. 2. Acetate and citrate content of the liver and kidneys after oral and intraperitoneal administration. Acetate: kidneys ▲, △; liver ■, □; citrate: kidneys ○, liver ●.



1 - Acetate and citrate content per g fresh weight.

The acetate content of the kidneys (Fig. 2) increases after, administration within the time intervals studied. At every time moment of the test, a higher acetate content was found in the kidneys than in the liver; 60 min after oral application, per gram of organ fresh weight, 0.8 micromoles were found in the liver and 1.4 micromoles in the kidneys. This difference in content is understandable in view of the established high conversion rate of acetate in the liver. The urine analysis (Fig. 3) showed a higher acetate content during the first two days after the start of the test. When compared with the high excretion rates of citrate (1), the measured excretion of acetate in urine (1.2 micromoles/ml) within the first 24 hours after administration, however, must be considered as relatively low.

Fig. 3. Acetate content in urine after oral administration.



1 - Acetate content per ml.

Tests with citric acid, carried out for comparison purposes, could be performed only by administering this compound perorally. Intraperitoneal administration of citrate in the concentration used here (125 mg) caused, within 5 minutes, strongly expressed tetanic cramp conditions, which resulted in the death of the animals (cf., in this case, also (1)).

In the liver, after administration, only a surprisingly slight increase of the citrate values is noted (Fig. 2). In the kidneys, the highest citrate content can be observed already after 30 min. This is in agreement with our studies (1), which showed that orally administered citrate is excreted at a surprisingly high rate in the urine within 2 hours after administration. Studies performed by Martin et al. (11) and other authors have also shown that the bulk of blood citrate is converted by the kidneys.

Summary

Acetic and citric acids, when introduced from the outside into the body or even per os or intraperitoneally in unphysiologically high doses, are utilized within a short time in the animal body. At the same time, there is no long-lasting increase in the content of these compounds in the blood and liver; 1-2 hours after administration, the citrate and acetate content of blood is largely normal again.

The liver shows a high absorption capacity for acetate and citrate. The sudden increase in blood acetate values, caused by intraperitoneal excess supply, is not followed by a corresponding course of the curve showing acetate values in the liver. The high acetate conversion of the liver, established here, is discussed.

On the basis of the results reported here, there are no valid reasons to support the occasionally expressed opinion that acetic acid supplied with food might constitute a burden for the organism.

Bibliography

cf. this journal

1. Medzihradsky, F., u. W. Lamprecht (Diese Z.) 130, 171 (1966).
2. Lundquist, F., H. Fugmann u. H. Rasmussen: Biochem. J. 80, 393 (1961).
3. Bratton, A. C., u. E. Marshall: J. biol. Chem. 128, 537 (1939).
4. Medzihradsky, F., u. W. Lamprecht: Hoppe-Seyler's Z. physiol. Chem. (im Druck). in press p.
5. Natelson, S., J. B. Pincus, u. J. K. Lugovoy: J. biol. Chem. 175, 745 (1948).
6. Elliot, K. A. C.: Methods in Enzymology, Bd III, S. 426. New York: Academic Press 1957.
7. Hochheuser, W., H. Weiss u. O. Wieland: Z. klin. Chem. 2, 175 (1964).
8. Forsander, O. A., N. Räihä u. H. Suomalainen: Hoppe-Seyler's Z. physiol. Chem. 318, 1 (1960). Tof.
9. Lundquist, F.: Nature (Lond.) 193, 579 (1962).
10. Dowden, D. R., D. R. Jacobson: Nature (Lond.) 188, 148 (1960).
11. Martin, G. R., H. E. Firschein, B. J. Muliyan u. W. F. Neuman: J. Amer. chem. Soc. 80, 6201 (1958).

Z. Lebensm.-Unters. Forsch. 131(4): 221-5, (1966)

Stoffwechseluntersuchungen mit Essig-, Milch- und Citronensäure

*II. Mitteilung**

Verteilung von zugeführter Essig- und Citronensäure im Tierkörper

Von

FEDOR MEDZIHRADSKY und WALTHER LAMPRECHT

Unter experimenteller Mitarbeit von MARIA REHLING

Mitteilung aus dem Biochemischen Laboratorium der Technischen Hochschule München

Mit 3 Textabbildungen

(Eingegangen am 24. September 1965)

Die vorliegende Arbeit wurde im Rahmen von Untersuchungen mit Essig-, Milch- und Citronensäure mit dem Ziel durchgeführt, das Stoffwechselgeschehen dieser, dem Organismus zugeführten Säuren aufzuklären. Über den Einfluß der genannten alimentären Säuren auf metabolische Zwischenstoffkonzentrationen im Tierkörper, ihre Ausscheidungsrate und über gluconeogenetische Eigenschaften wurde in einer vorangegangenen Arbeit berichtet (1). Die Ergebnisse dieser Versuche zeigten u. a., daß nach oralen Gaben von ^{14}C-markierter Essig- oder Citronensäure in kurzer Zeit hohe $^{14}CO_2$-Raten in der Atemluft der Tiere resultieren. Die maximale $^{14}CO_2$-Ausatmungsrate liegt bei Citronen- und Essigsäure in der 3. bzw. 5. Std nach der per os-Verabreichung, mit 20 bzw. 15% der zugeführten Aktivität.

In den vorliegenden Untersuchungen wird die zeitliche Verteilung von Essig- und Citronensäure in Blut, Leber und Niere nach peroraler und intraperitonealer Verabreichung gemessen**.

Experimenteller Teil

1. Material

Für sämtliche Versuche wurden weibliche Reinzuchtratten mit einem Gewicht von ca. 200 g verwendet. Die Tiere wurden mit Standardfutter „Altromin R" ernährt. 6 Std vor Versuchsbeginn wurde das Futter entzogen. Der zur Acetatbestimmung erforderliche Taubenleberextrakt wurde uns freundlicherweise von Herrn Prof. Dr. O. WIELAND zur Verfügung gestellt***. Coenzym A und ATP sind Handelspräparate der Fa. Boehringer & Söhne, die übrigen Chemikalien sind vom Reinheitsgrad pro anal.

2. Entnahme und Aufarbeitung der Proben

Die Verabreichung der genannten Säuren erfolgte in getrennten Versuchsreihen per Schlundsonde (300 mg Essig- bzw. Citronensäure in 1 ml einer mit NaOH auf pH 4 eingestellten Lösung) bzw. intraperitoneal (125 mg der Na-Salze der Säuren).

Blutproben wurden in festgelegten Zeitabständen (vgl. Abb. 1) durch Herzpunktion am lebenden Tier entnommen[1]. Die Proben wurden in der Kälte (2–4° C) mit vorgelegter 6%iger

* I. Mitteilung vgl. (1).

** Herrn Dr. F. MARTENS sind wir für anregende Diskussionen zu Dank verpflichtet.

*** Herrn Dr. D. HEPP danken wir für das freundliche Entgegenkommen und die Ratschläge bei der Durchführung der enzymatisch-colorimetrischen Acetatbestimmung.

[1] Die Herzpunktion wurde in Zusammenarbeit mit Herrn cand. med. P. BALDE durchgeführt wofür wir besonders danken.

$HClO_4$ enteiweißt, 10 min bei 1000 × g zentrifugiert, dekantiert, der Rückstand mit Perchlorsäure aufgenommen und erneut zentrifugiert. Die vereinigten Überstände wurden mit K_2CO_3 auf pH 7 neutralisiert und 30 min bei 0° C stehengelassen. Die Lösungen, vom $KClO_4$-Niederschlag abdekantiert, wurden für die Bestimmung der Metabolitkonzentrationen verwendet.

Die Organproben (Leber und Niere) wurden nach dem Dekapitieren der Tiere in kürzester Zeit (innerhalb 1 min) in flüssigen Stickstoff gebracht, anschließend in der Reibschale fein pulverisiert und in der Kälte (2–4° C) in vorgelegte Perchlorsäure eingewogen. Die weitere Behandlung erfolgte wie oben beschrieben.

In getrennten Versuchen wurde die Ausscheidung von Acetat im Harn nach entsprechend peroraler Belastung untersucht. Nach erfolgter Schlundsonde-Gabe wurden die Tiere im Stoffwechselkäfig bei ausschließlicher Trinkwasserversorgung 72 Std belassen und in dieser Zeit Harnproben aufgefangen. Diese wurden mit Perchlorsäure im Verhältnis 1 : 1 enteiweißt, zentrifugiert und im neutralisierten Überstand die Substratanalysen durchgeführt.

3. Acetat- und Citratbestimmung

Acetat wurde nach der Methode von LUNDQVIST (2) bestimmt. Nach diesem Verfahren werden die biologischen Perchlorsäureextrakte einer Mikrodiffusion nach CONWAY unterzogen, wobei die Essigsäure in vorgelegte Natronlauge überführt wird. Die Bestimmung selbst erfolgte enzymatisch-optisch mit Hilfe eines Taubenleberextraktes, der mit Coenzym A und ATP, Acetat zu Acetyl-CoA umwandelt, worauf vorgelegtes Sulfanilamid acetyliert wird. Das unverbrauchte Sulfanilamid wird colorimetrisch bestimmt (3) Die umgesetzte Menge des Reagens ist der Acetatkonzentration proportional.

Die außerordentlich zeitraubende und auch mühevolle Durchführung der enzymatischen Acetatbestimmung führte uns zu der Ausarbeitung einer routinemäßig durchführbaren gaschromatographischen Bestimmung von Essigsäure in Blut und Organen [MEDZIHRADSKY und LAMPRECHT (4)].

Die Bestimmung von Citronensäure erfolgte colorimetrisch nach NATELSON und Mitarb. bzw. ELLIOT (5, 6). In diesem Verfahren wird Citronensäure mit $KMnO_4$ und KBr in Pentabromaceton überführt. Diese Verbindung liefert mit Thioharnstoff einen Komplex, dessen Farbintensität bei 430 nm bestimmt wird.

Ergebnisse und Diskussion

Die in den Abbildungen dargestellten Ergebnisse sind Mittelwerte aus jeweils 2–4 Versuchen.

Wie in der Abb. 1 ersichtlich, verursacht sowohl peroral wie intraportal verabreichtes Acetat nur eine kurzzeitige Erhöhung des Acetatgehaltes im Blut. Das intraperitoneal als Depotsubstanz dem Tierkörper angebotene Acetat tritt schon nach 15 min mit Maximalwerten von 12 µmol/g Frischgewicht (Fr.-Gew.) im Blut auf.

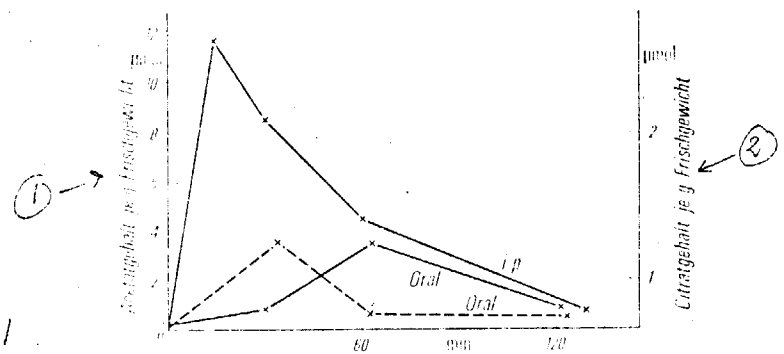


Abb. 1. *Acetat- und Citratgehalt in Blut nach oraler und intraperitonealer Verabreichung.* Acetat: ×——×; Citrat: ×- - - - -×

Bei oraler Applikation werden infolge der dazwischenliegenden Resorption Höchstwerte (3.5 µmol/g Fr.-Gew.) erst 60 min nach Verabreichung erreicht. Trotz dieser zeitlichen wie auch mengenmäßigen Unterschiede der Acetatgehalte nach oraler

(300 mg) und intraperitonealen Gaben (125 mg) sind die Abklingzeiten dieser Verbindung im Blut in beiden Fällen auffallend ähnlich. 2 Std nach der Verabreichung ist der versuchsbedingte Acetatanstieg im Blut wieder abgefallen. Auch die sprunghaft eingetretene Erhöhung der Acetatwerte im Blut nach dem hohen intraperitonealen Angebot fällt innerhalb 1 Std auf den nach oralen Gaben erreichten Höchstwert ab. Von diesem Zeitpunkt an tritt Acetat in vergleichbaren Raten aus dem Blutkreislauf aus.

Die Citratgehalte im Blut (Abb. 1) zeigen einen ähnlichen zeitlichen Verlauf wie Acetat. In Übereinstimmung mit früheren Ergebnissen (1) wird Citronensäure gegenüber Essigsäure schneller durch den Magen-Darm Trakt in die Blutbahn eingeschleust, wie auch der Austritt aus dem Blut eher erfolgt. Maximalgehalte an Citrat (0,9 µmol/g Fr.-Gew.) werden schon 30 min nach Verabreichung erreicht. Nach 60 min ist dieser Gehaltsanstieg wieder normalisiert.

Die Normalwerte im Blut betragen 0,311 µmol Acetat bzw. 0,034 µmol Citrat jeweils pro g Fr.-Gew. In der Leber und Niere finden sich pro g Fr.-Gew. 0,311 und 0,368 µmol Acetat bzw. 0,147 und 0,179 µmol Citrat.

Die Änderung der Acetatgehalte in Leber nach Belastung entsprechen dem Verlauf der Acetat-Blutwerte (Abb. 2). 60 min nach der Verabreichung wird mehr Acetat von der Leber umgesetzt als aufgenommen. Dabei erreicht der Acetatgehalt in diesem Organ zu keinem Zeitpunkt einen auch nur vergleichbar so eindeutigen Anstieg wie im Blut. Der höchste Wert läßt sich nach intraperitonealen Gaben feststellen und beträgt ca. das 3fache des Normalwertes. Offenbar wird nach dem in diesen Versuchen festgestellten kurzen Verbleib von Acetat in Blut dieses Substrat nach der Aufnahme in die Leber von diesem Organ in hohen Raten umgesetzt. Dieser Befund entspricht den Versuchsergebnissen von Hochheuser, Weiss und Wieland (7), wonach von der isolierten Rattenleber bei Perfusion hohe Acetatmengen aufgenommen werden.

Andererseits erhielten Forsander, Räihä und Suomalainen (8) bei Leberdurchströmung mit ^{14}C-Äthanol nur niedrige $^{14}CO_2$-Raten. Die $^{14}CO_2$-Ausbeute erhöhte sich jedoch beträchtlich, wenn auch die hinteren Extremitäten der Tiere mit Blut durchströmt wurden. Ähnliche Ergebnisse eigener Untersuchungen lassen Lundquist (9) zu dem Schluß kommen, daß Acetat vornehmlich von den peripheren Organen, in diesen jedoch bevorzugt umgesetzt wird. Die Erklärung dieser Primatstellung von Acetat, wonach durch dessen Aktivierung an der Acetatthiokinase ein großer Teil des der Zelle zur Verfügung stehenden Coenzyms A anderen Enzymsystemen entzogen wird, läßt sich auch zum Verständnis einer weiteren experimentellen Beobachtung heranziehen. Die in Tierversuchen nach i. v. Acetatgaben eintretende Appetitlosigkeit (Sattheitsgefühl) (10), könnte durch eine CoA-Mangel bedingte Behinderung des Fettsäureabbaues hervorgerufen werden.

In anderen Untersuchungen mit ^{14}C-markierten Verbindungen (1) konnten wir für von außen dem Tierkörper zugeführte Essig- und Citronensäure feststellen, daß sie über die Reaktionsfolgen des Citronensäurecyclus gluconeogenetisch verwertet werden können.

Der Acetatgehalt der Niere (Abb. 2) steigt nach Verabreichung innerhalb der untersuchten Zeiträume an. Zu jedem Zeitpunkt des Versuchs wurden in der Niere höhere Acetatgehalte als in der Leber festgestellt. 60 min nach der oralen Applikation finden sich pro g Organfrischgewicht in Leber 0,8 µmol, in Niere 1,4 µmol. Dieser Gehaltsunterschied wird durch die festgestellte hohe Umsatzrate von Acetat

in der Leber verständlich. Die Harnanalyse (Abb. 3) ergab im Laufe der ersten beiden Tage nach Versuchsbeginn einen erhöhten Acetatgehalt. Verglichen mit den hohen Ausscheidungsraten von Citrat (1), läßt sich die festgestellte Ausscheidung von Acetat in Harn mit 1,2 µmol/ml innerhalb der ersten 24 Std nach der Verabreichung jedoch als relativ niedrig bezeichnen.

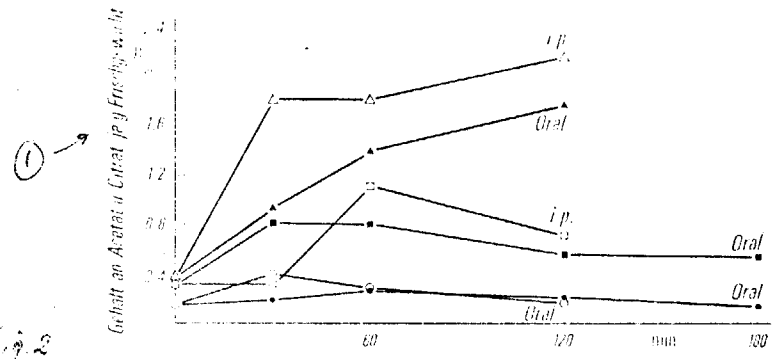


Abb. 2. *Acetat- und Citratgehalt in Leber und Niere nach oraler und intraperitonealer Verabreichung.* Acetat: Niere ▲, △; Leber ■, □; Citrat: Niere ○, Leber ●

Die zum Vergleich durchgeführten Versuche mit Citronensäure konnten nur durch peorale Verabreichung dieser Verbindung erfolgen. Intraperitoneal verabreichtes Citrat in der hier angewandten Konzentration (125 mg) verursacht innerhalb von 5 min stark ausgeprägte tetanische Krampfzustände, die zum Tode der Tiere führten (vgl. dazu auch (1)).

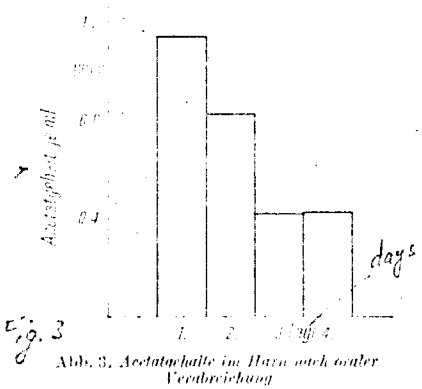


Abb. 3. *Acetatgehalte im Harn nach oraler Verabreichung*

In der Leber wird nach Belastung nur eine auffallend geringe Erhöhung der Citratwerte erreicht (Abb. 2). In der Niere läßt sich schon nach 30 min der höchste Citratgehalt feststellen. Dies entspricht unseren Untersuchungen (1), wonach oral verabreichtes Citrat innerhalb 2 Std nach der Gabe in auffallend hohen Raten im Harn ausgeschieden wird. Untersuchungen von Martin und Mitarb. (11) und anderen Autoren haben ebenfalls gezeigt, daß das Blutcitrat zum überwiegenden Teil von der Niere umgesetzt wird.

Zusammenfassung

Extracorporal und sogar in unphysiologisch hohen Dosen per os oder intraperitoneal zugeführte Essig- und Citronensäure wird im Tierkörper in kurzer Zeit verwertet. Dabei tritt keine länger anhaltende Erhöhung der Gehalte dieser Verbindungen in Blut und Leber auf. 1 bzw. 2 Std nach der Verabreichung ist der Citrat- bzw. Acetatgehalt im Blut weitgehend normalisiert.

Die Leber zeigt eine hohe Aufnahmekapacität für Acetat und Citrat. Dem im Blut durch intraperitoneales Überangebot hervorgerufenen sprunghaften Anstieg der Acetatwerte folgt kein entsprechender Kurvenverlauf in der Leber.

Der festgestellte hohe Acetatumsatz der Leber wird diskutiert.

Für die gelegentlich anzutreffende Ansicht, in der Nahrung zugeführte Essigsäure könne eine Belastung des Organismus bedeuten, finden sich auf Grund der vorgelegten Ergebnisse keine Anhaltspunkte.

cf. this journal

Literatur

1. Medzihradsky, F., u. W. Lamprecht: Diese Z. 130, 171 (1966).
2. Lundquist, F., H. Fugmann u. H. Rasmussen: Biochem. J. 80, 393 (1961).
3. Bratton, A. C., u. E. Marshall: J. biol. Chem. 128, 537 (1939).
4. Medzihradsky, F., u. W. Lamprecht: Hoppe-Seyler's Z. physiol. Chem. (im Druck). in press
5. Natelson, S., J. B. Pincus, u. J. K. Lucovoy: J. biol. Chem. 175, 745 (1948).
6. Elliot, K. A. C.: Methods in Enzymology. Bd. III, S. 426. New York: Academic Press 1957.
7. Hochheuser, W., H. Weiss u. O. Wieland: Z. klin. Chem. 2, 175 (1964).
8. Forsander, O. A., N. Räihä u. H. Suomalainen: Hoppe-Seyler's Z. physiol. Chem. 318, 1 (1960).
9. Lundquist, F.: Nature (Lond.) 193, 579 (1962).
10. Dowden, D. R., D. R. Jacobson: Nature (Lond.) 188, 148 (1960).
11. Martin, G. R., H. E. Firschein, B. J. Mulivan u. W. F. Neuman: J. Amer. chem. Soc. 80, 6201 (1958).

GROWTH OF *STAPHYLOCOCCUS AUREUS* IN ACIDIFIED PASTEURIZED MILK[1]

T.-E. Minor and E. H. Marth

Department of Food Science and The Food Research Institute
University of Wisconsin
Madison, Wisconsin 53706

(Received for publication July 13, 1970)

Abstract

The effect of gradually reducing the pH of pasteurized milk with acetic, citric, hydrochloric, lactic, and phosphoric acids over periods of 4, 8, and 12 hr on growth of *Staphylococcus aureus* 100 in this substrate was determined. In addition, 1:1 mixtures of lactic acid and each of the other acids, and of acetic and citric acids were evaluated for their effect on growth of this organism. To achieve a 90% reduction in growth over a 12 hr period, a final pH value of 5.2 was required for acetic, 4.9 for lactic, 4.7 for phosphoric and citric, and 4.6 for hydrochloric acid. A 99% reduction during a 12 hr period was obtained with a final pH value of 5.0 for acetic, 4.6 for lactic, 4.5 for citric, 4.1 for phosphoric, and 4.0 for hydrochloric acid. A pH value of 3.3 was required for a 99.9% reduction with hydrochloric acid, whereas the same effect was produced at a pH value of 4.9 with acetic acid. Correspondingly lower pH values were required to inhibit growth within 8 and 4 hr periods. Mixtures of acids adjusted to pH values at the borderline for growth (12 hr period) exhibited neither synergistic nor antagonistic effects between two acids.

Several reports in recent years have alluded to a direct relationship between pH and degree of growth by or survival of *Staphylococcus aureus* in dairy products. It is widely accepted that starter culture growth and activity in fermented dairy products has an inhibitory effect on *S. aureus* (*1, 2, 4*) but the contribution by pH has not always been clearly demonstrated. Zehren and Zehren (*8*) examined manufacturing records for 378 vats of cheese of which 59 were found to contain staphylococcal enterotoxin A and the authors concluded that a relationship existed between subnormal acid development and toxin production. Growth of staphylococci in various kinds of cheese was investigated by Tuckey et al. (*5*) who suggested that a pH value above rather than below 5.3 was more favorable for proliferation of the bacteria. According to Walker et al. (*6*), no correlation existed between staphylococcal populations and pH values of 5.1 to 5.75 in Colby cheese during ripening for 120 days.

The present study was conducted to define more clearly the relationship between acidity in pasteurized milk and growth of *S. aureus*.

[1]Published with the approval of the Director of the Research Division of the College of Agricultural and Life Sciences University of Wisconsin.

Materials and Methods

Two series of experiments were performed. The first dealt with the effect on staphylococcal growth of gradually adding to milk a selected quantity of a single acid over a 4, 8, and 12 hr period. The second series involved a comparison of the effect of two acids acting independently with that obtained when the acids were mixed (1:1) and added gradually over a 12 hr period.

Apparatus

Each experiment was performed using the apparatus illustrated in Fig. 1. The basic unit of the system is a 2-liter, jacketed, three-necked flask (ST 24/40 joints) equipped with a 6 mm bore, stopcock for draining (Corning Glass, No. 94602). The center neck is fitted with a mercury-seal stirrer bearing and a glass stirring rod (9 mm in diameter) with an attached Teflon blade (93 mm wide: Kontes, No. K-786536). The stirrer is driven by a 55 watt, 1550 rpm Talboy T-line motor (No. 105). The neck on the right of the flask is equipped with a 25 ml burette and the neck on the left is fitted with a thermometer (and also serves as a port through which the milk is added and small samples removed). Four flasks are employed in each experiment, permitting the simultaneous study of three variables while one of the flasks serves as the vessel for a control. The flasks rest on padded rings connected to a 36 by 30 inch frame. Each flask is held firmly in place by two large clamps.

The stirring motors are all connected to a single rheostat. Stirring speeds are regulated by measuring and controlling the total amperage output from the rheostat. The relation between amperage output and rpm of the revolving stirrer shafts has been determined. A stirring rate of 300 rpm (with a variation of ± 10% between flasks) was used throughout this study.

Temperature-controlled water is provided by a Tecam Temp Unit (A.S. LaPine, No. 414-60) appropriately attached to a large stainless steel beaker. The water is circulated to the flasks through 0.5 inch (inside diameter) tubing connected to an Eastern centrifugal pump driven by a 1/15 hp, 5,000 rpm motor operating at nearly full capacity. Rather than connect the flasks in series, we have provided a single tube with branches to simultaneously feed water to the bottom of the jackets of all the flasks and a single branched tube to return water from the tops of the jackets to the steel beaker. Since circulation of water is rapid, we have been unable to detect differences in temperature between flasks.

Between experiments flasks were cleaned-in-place, then sanitized-in-place with a 200 ppm hypochlorite solution, and allowed to drain dry overnight before use.

Addition of acids

The following acids were used: acetic, glacial, A.C.S. reagent (Allied); citric, granular, analytical reagent (Mallinckrodt); hydrochloric, reagent (DuPont); lactic, USP 85% (Mallinckrodt); and phosphoric, A.C.S. reagent (Allied).

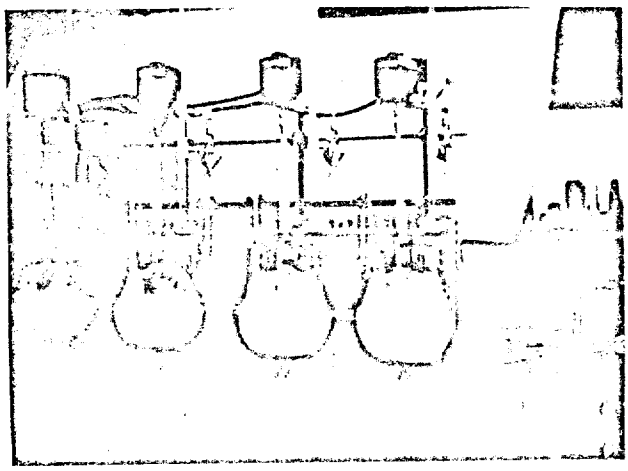


Figure 1. Apparatus used for growth of *Staphylococcus aureus* in pasteurized milk.

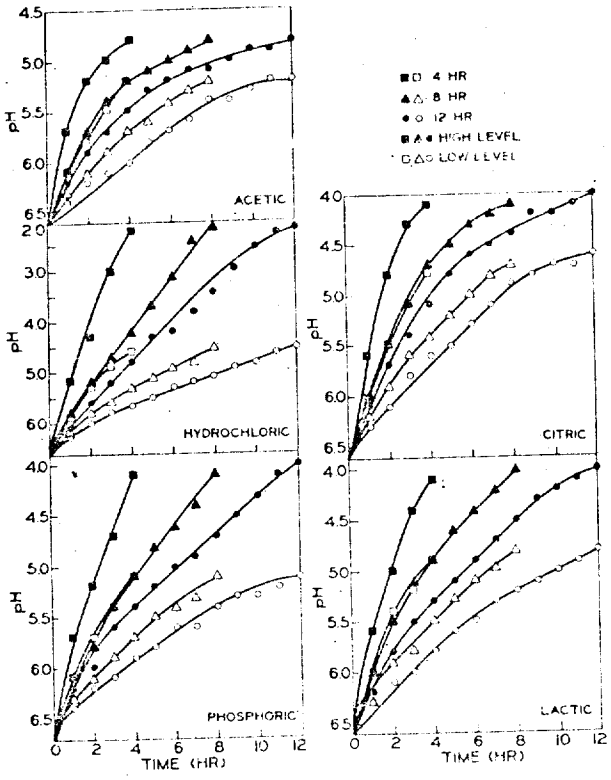


Figure 2. Change in pH of pasteurized milk achieved through the addition of two levels of acetic, citric, hydrochloric, phosphoric, and lactic acids independently over periods of 4, 8, and 12 hr.

Pasteurized homogenized whole milk of excellent bacteriological quality (Standard Plate Count of approximately 300 per milliliter) was obtained from the University of Wisconsin Dairy plant on the day preceding each experiment. One quart was used per flask.

Acids were prepared as solutions in distilled water at concentrations so that the desired quantity could be distributed over the course of the experiment in 6.5 ml increments added to milk every 15 min. The acidic solutions were steamed 15 min to reduce microbial contamination and dispensed from autoclaved 25 ml burettes. Steamed distilled water was added to one of the flasks in each experiment to serve as a control.

Titratable acidity and pH were monitored hourly in the first series of experiments and every 2 hr in the second series.

Culture

A culture of *S. aureus* 100 (a good enterotoxin A producer) was obtained from Dr. K. F. Weiss (Food Research Institute, University of Wisconsin). Cultures were carried on Brain Heart Infusion agar (Difco) slants for up to three months and were transferred twice in Brain Heart Infusion broth before use. All four flasks in each experiment were inoculated with 5 ml of a 1:100 dilution of an 18 ± 1 hr broth culture in phosphate buffer. This resulted in an initial plate count of approximately 2 x 10^4 cells per milliliter in each flask. An incubation temperature of 37 C was provided for optimum growth of *S. aureus*.

Determination of growth

Plate counts were performed according to recommendations of *Standard Methods for the Examination of Dairy Products* (7) every 4 hr and in the first series of experiments a count was determined after 2 hr. Plates were poured with Mannitol Salt Agar (Difco) and incubated at 37 C for 2 days. Mannitol-fermenting colonies of proper size and appearance were reported as *S. aureus* and occasionally were confirmed by microscopic examination.

The natural flora of the milk interfered with enumeration of staphylococci when Staphylococcus 110 Medium (Difco)

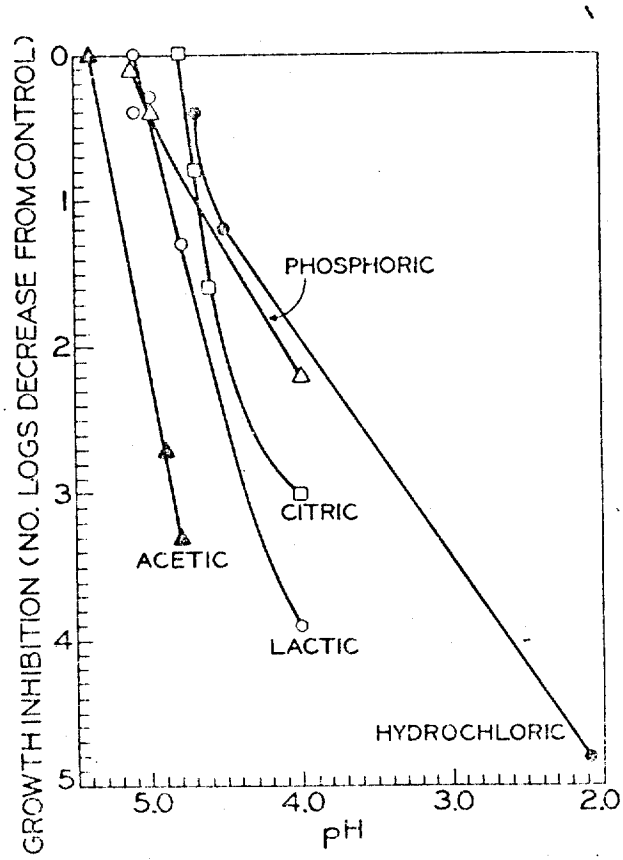


Figure 3. Relationship between terminal pH value of pasteurized milk fortified with different acids and inhibition of *Staphylococcus aureus*.

TABLE 1. GROWTH OF *Staphylococcus aureus* 100 IN PASTEURIZED MILK ACIDIFIED WITH DIFFERENT ACIDS DURING 4 HOURS OF INCUBATION AT 37 C[1]

Acid	Final pH	Final Per cent added acid	Decrease in growth from control No. logs[2] 2 hr	Decrease in growth from control No. logs[2] 4 hr	Difference from initial number No. logs[3] 2 hr	Difference from initial number No. logs[3] 4 hr
Acetic	5.2	0.22	0.2	0.9	0.1	0.1
	4.8	0.43	0.2	0.8	0.1	0.2
Citric	4.8	1.03	0.3	0.7	0.2	0.0
	4.1	1.97	0.6	1.0	0.0	0.1
Hyrdochloric	4.6	0.17	0.3	0.7	0.1	0.4
	2.2	0.42	0.4	1.5	0.1	−0.4
Lactic	4.9	0.41	0.1	0.3	0.3	0.8
	4.1	0.70	0.3	0.9	0.1	0.2
Phosphoric	5.1	0.60	0.1	0.6	0.1	0.3
	4.1	1.20	0.4	1.0	0.4	0.2

[1]Inoculated to contain 2.1 (± 0.7) x 10^4 staphylococci per milliliter.
[2]Number per milliliter in control: 2 hr: 5.2 (±2.1) x 10^5; 4 hr: 6.6 (± 1.5) x 10^5.
[3]Positive values indicate an increase in numbers and negative values a decrease.

TABLE 2. GROWTH OF *Staphylococcus aureus* 100 IN PASTEURIZED MILK ACIDIFIED WITH DIFFERENT ACIDS DURING 8 HOURS OF INCUBATION AT 37 C[1]

Acid	Final pH	Final Per cent added acid	Decrease in growth from control No. logs[2] 2 hr	Decrease in growth from control No. logs[2] 4 hr	Decrease in growth from control No. logs[2] 8 hr	Difference from initial number No. logs[3] 2 hr	Difference from initial number No. logs[3] 4 hr	Difference from initial number No. logs[3] 8 hr
Acetic	5.2	0.23	0.2	0.7	1.9	0.0	0.2	0.8
	4.8	0.43	0.2	0.8	2.9	0.1	0.2	0.3
Citric	4.7	1.11	0.3	0.0	1.8	0.2	0.4	0.7
	4.1	2.29	0.3	0.8	2.0	0.1	0.2	0.7
Hydrochloric	4.5	0.16	0.0	0.1	0.9	0.4	1.0	2.1
	2.1	0.43	0.3	0.8	3.9	0.2	0.2	−0.6
Lactic	4.8	0.41	0.1	0.1	0.2	0.2	0.9	2.1
	4.0	0.75	0.2	0.8	3.0	0.1	0.2	0.3
Phosphoric	5.1	0.60	0.1	0.3	0.7	0.1	0.6	1.8
	4.1	1.20	0.4	0.2	3.0	0.4	1.0	1.0

[1]Inoculated to contain 2.1 (± 0.7) x 10^4 staphylococci per milliliter.
[2]Number per milliliter in control: 2 hr: 5.2 (± 2.1 x 10^5; 4 hr: 6.6 (± 1.5) x 10^5; 8 hr: 9.3 (± 12) x 10^7.
[3]Positive values indicate an increase in numbers and negative values a decrease.

was used but created no problem with Mannitol Salt Agar. It also has been reported that both media recover staphylococci from cheese with equal efficiency (5). Consequently Mannitol Salt Agar was adopted for use throughout this study.

RESULTS AND DISCUSSIONS

Addition of a single acid

Figure 2 presents data on the change in pH achieved in milk through the addition of two levels of different acids over a 4, 8, or 12 hr period. The effect of these additions on the growth of staphylococci is detailed by data in Tables 1, 2, and 3.

Since the staphylococci increased by little more than one log in the control after 4 hr of incubation, it was difficult to demonstrate a marked difference in growth during a 4 hr period when acids were added (Table 1). If one considers as significant only those differences of one log or greater, then only the high levels of citric (1.97%), hydrochloric (0.42%), and phosphoric (1.20%) acids were effective [but both levels of acetic and the high level of lactic acid (0.7%) were on the borderline of significance] in retarding growth of *S. aureus*.

Substantial differences were more easily observed when acids were added over an 8 hr period (Table 2). Both levels of acetic and citric and the high levels of hydrochloric (0.43%), lactic (0.75%), and phosphoric (1.2%) acids were highly effective in inhibiting *S. aureus*. None of the levels produced greater than a one log difference after 2 or 4 hr of incubation.

Both levels of all acids, except a low concentration of phosphoric (0.62%), produced substantial differences in growth after 12 hr of incubation (Table

TABLE 3. GROWTH OF *Staphylococcus aureus* 100 IN PASTEURIZED MILK ACIDIFIED WITH DIFFERENT ACIDS DURING 12 HOURS OF INCUBATION AT 37 C[1]

Acid	Final pH	Final Per cent added acid	Decrease in growth from control—No. logs[2] 2 hr	4 hr	8 hr	12 hr	Difference from initial numbers—No. logs[3] 2 hr	4 hr	8 hr	12 hr
Acetic	5.2	0.23	0.0	0.2	0.6	1.9	0.2	0.7	0.8	2.1
	4.8	0.45	0.1	0.7	2.3	3.3	0.2	0.3	0.9	0.9
Citric	4.6	1.26	0.3	0.0	0.2	1.6	0.2	0.7	2.2	2.4
	4.0	2.58	0.2	0.4	1.8	3.0	0.2	0.6	0.8	1.2
Hydrochloric	4.5	0.18	0.0	0.1	0.0	1.2	0.6	1.1	3.1	3.2
	2.1	0.43	0.2	0.2	2.4	4.8	0.3	0.8	0.9	−0.7
Lactic	4.8	0.42	(0.1)[4]	0.4	0.6	1.3	0.4	0.6	1.8	2.7
	4.0	0.76	0.2	0.7	2.3	3.9	0.3	0.3	1.0	0.4
Phosphoric	5.1	0.62	0.0	0.2	0.0	0.1	0.2	0.7	2.6	3.6
	4.0	1.26	0.3	0.1	1.8	2.2	0.4	1.0	2.2	2.4

[1]Inoculated to contain 2.1 (± 0.7) x 10^4 staphylococci per milliliter.
[2]Number per milliliter in control: 2 hr: 5.2 (± 2.1 x 10^4; 4 hr: 6.6 (± 1.5) x 10^5; 8 hr: 9.3 (± 12) x 10^5; 12 hr: 3.7 (± 1.5) x 10^6.
[3]Positive values indicate an increase in numbers and negative values a decrease.
[4]An increase from the control.

3). Only the high levels of all the acids showed a marked reduction in growth at the 8 hr point. There were no differences among the acids at 2 or 4 hr.

Addition of mixtures of acids

Mixtures of acids, for the most part, were evaluated at pH values on the borderline of affecting staphylococcal growth (Table 4). This series of experiments also was used to determine the reproducibility of data obtained in the initial trials. Neither synergistic nor antagonistic effects were demonstrated with any of the mixtures which were evaluated.

Relationships between pH and growth

The relationship between pH and growth inhibition is summarized in Fig. 3 from the data presented in Tables 3 and 4 (12 hr periods). Acetic acid had the greatest effect on growth in that the threshold for minimum inhibition was pH 5.3-5.4 and a pH value of 4.8 produced more than 99.9% reduction in growth. The threshold for growth-inhibition by lactic and phosphoric acid was pH 5.1-5.2 followed by citric acid at pH 4.8 and hydrochloric slightly below citric. Whereas these thresholds were closely grouped, differences were greater at lower pH values. To achieve a 90% reduction in growth, a final pH value of 5.2 was required for acetic, 4.9 for lactic, 4.7 for phosphoric and citric, and 4.6 for hydrochloric acid. To achieve a 99% reduction in growth, a final pH of 5.0 was required for acetic, 4.6 for lactic, 4.5 for citric, 4.1 for phosphoric, and 4.0 for hydrochloric acid. A pH value of 3.3 was required for a 99.9% reduction with hydrochloric while the same effect was produced at pH 4.9 with acetic acid. The organic acids were thus more effective than the mineral acids.

Results of this study demonstrated that the pH of cultured dairy products may have to approach a value of 4 by the end of the fermentation in order to achieve control of staphylococcal growth during 4 hr fermentations; but, then, growth of the organism is slow during this period of incubation. A final pH value of 4.5 may be effective for control during 8 hr fermentations and a final pH value slightly below 5 may be sufficient for control during 12 hr fermentations. When acids are used for acidification in lieu of starter cultures, acetic acid appears as the best choice for control of staphylococci and hydrochloric the least satisfactory of those acids tested using the conditions established in our experiments. Direct acidification as currently practiced, however, is completed in less than 15-30 min and the pH is rapidly reduced to 4.6 to 5.6. These two conditions minimize the opportunity for staphylococcal growth for more than when starter cultures are used. The effectiveness of lactic acid at higher pH values can be greatly extended by adding acetic acid, but mixtures of other acids would be of little additional benefit.

Subramanian and Marth *(3)* have conducted studies similar to those described in this paper using *Salmonella typhimurium*. They found citric acid to be most inhibitory to the organism followed in order by lactic and hydrochloric acids when added gradually over a 16 hr period to final pH values of 4.5, 4.25, and 4.0, respectively, in skimmilk held at 37 C.

Results of this work justify further investigations on how other factors together with pH serve to inhibit growth of and enterotoxin production by *S. aureus*.

TABLE 4. GROWTH OF *Staphylococcus aureus* 100 IN PASTEURIZED MILK ACIDIFIED WITH DIFFERENT ACIDS AND MIXTURES OF ACIDS DURING 12 HOURS OF INCUBATION AT 37 C[1]

Acid	Final pH	Final Per cent added acid	Decrease in growth from control No. logs[2] 4 hr	8 hr	12 hr	Difference from initial numbers No. logs[3] 4 hr	8 hr	12 hr
Acetic	5.4	0.22	0.1	0.1	0.0	1.0	4.0	4.1
Citric	4.8	0.99	0.1	0.1	0.0	1.0	4.0	4.1
1:1 Mixture	5.1	—	0.2	0.1	0.1	0.9	4.0	4.0
Lactic	5.1	0.35	0.4	0.7	0.0	0.9	2.1	4.0
Acetic	4.9	0.31	0.6	1.7	2.7	0.7	1.0	1.2
1:1 Mixture	5.0	—	0.4	0.8	1.6	0.9	1.9	2.3
Lactic	5.0	0.39	0.1	0.7	0.3	1.1	3.3	4.0
Citric	4.7	1.10	0.1	0.9	0.8	1.1	3.1	3.6
1:1 Mixture	4.8	—	0.1	0.9	0.3	1.1	3.1	4.0
Lactic	5.0	0.39	0.4	0.7	0.4	1.2	3.4	3.9
Hydrochloric	4.7	0.14	0.4	1.0	0.4	1.2	3.1	3.9
1:1 Mixture	4.8	—	0.4	0.9	0.3	1.2	3.2	4.0
Lactic	5.1	0.37	0.2	0.4	0.4	2.2	3.7	3.9
Phosphoric	5.0	0.83	0.3	0.7	0.4	2.1	3.5	3.9
1:1 Mixture	5.0	—	0.2	0.7	0.4	2.2	3.5	3.9

[1]Inoculated to contain 2.1 (± 0.7) x 10^3 staphylococci per milliliter.
[2]Number per milliliter in control: 4 hr: 6.6 (± 1.5) x 10^6; 8 hr: 9.3 (± 12) x 10^7; 12 hr: 3.7 (± 1.5) x 10^8.
[3]Positive values indicate an increase in numbers and negative values a decrease.

ACKNOWLEDGMENT

This work was supported in part by Public Health Service Grant No. FD00609-03 from the Food and Drug Administration.

REFERENCES

1. Kao, C. T., and W. C. Frazier. 1966. Effect of lactic acid bacteria on growth of *Staphylococcus aureus*. Appl. Microbiol. 14:251-255.

2. Reiter, B., B. G. Fewins, T. F. Fryer, and M. E. Sharpe. 1964. Factors affecting the multiplication and survival of coagulase positive staphylococci in Cheddar cheese. J. Dairy Res. 31:261-272.

3. Subramanian, C. S., and E. H. Marth. 1968. Multiplication of *Salmonella typhimurium* in skimmilk with and without added hydrochloric, lactic, and citric acids. J. Milk Food Technol. 31:323-326.

4. Takahashi, I., and C. K. Johns. 1959. *Staphylococcus aureus* in Cheddar cheese. J. Dairy Sci. 42:1032-1037.

5. Tuckey, S. L., M. E. Stiles, Z. J. Ordal, and L. D. Witter. 1964. Relation of cheese-making operations to survival and growth of *Staphylococcus aureus* in different varieties of cheese. J. Dairy Sci. 47:604-611.

6. Walker, G. C., L. G. Harmon, and C. M. Stine. 1961. Staphylococci in Colby cheese. J. Dairy Sci. 44:1272-1282.

7. Walter, W. G. (ed.). 1967. Standard methods for the examination of dairy products. 12th ed. Amer. Public Health Ass., Inc. New York.

8. Zehren, V. L., and V. F. Zehren. 1968. Relation of acid development during cheesemaking to development of staphylococcal enterotoxin. A. J. Dairy Sci. 51:645-649.

THE FUTURE OF EDIBLE OILS

(Continued from Page 515)

rather an oblique manner. The future deployment of edible oils as an ingredient in food stuffs manufactured by dairy processors is not up to the gods—but only to the politicians. A statesman-like, business-oriented position would be to rid the industry of these artificial restrictions that have not and will not operate to the best long-term interests of either the processor or the producer.

There are a host of reasons why but let me illustrate only two. A few years ago I was talking to an American, who pioneered the vegetable oil coffee whitener business. Originally his formula called for a vegetable oil and milk solids combination. When he discovered that this end-product contravened certain interstate commerce regulations, he went back to the drawing board and developed a formula totally void of any dairy product. This he could legally move interstate. He advised me that even if it became legal to revert to his original formula he would not do so. His estimate of the potential lost market to the producer of milk solids was astronomical and he only hoped that Canada would benefit by the short-sighted U.S. policy that resulted in the production of the totally non-dairy whitener—and lost a substantial market forever.

Or what about the impact of the elasticity of demand? This phase of market research is receiving a considerable amount of study at present—particularly in the U.S. The University of California has com-

(Continued on Page 523)

Nuclear-Med. 2(2): 165-172, 1961

In Vitro Incorporation of Sodium Acetate-l-C^{14} into Leukocyte Lipids of Normal and Leukaemic Subjects as a Function of Incubation Time

From the Department of Clinical Therapeutics of the University of Athens, Alexandra Hospital (Director: Prof. B. Malamos)

C. Miras, G. Lewis and J. Mantzos

In a previous paper the rate of the incorporation of acetate-l-C^{14} (CH_3COONa-l-C^{14}) into mixed lipids of leukaemic blood in vitro was studied. It was shown that when the patients were treated with alkylating agents or antimetabolites the incorporation during an incubation of 6 hours duration was markedly reduced. These studies were performed under the same conditions on blood of leukaemic subjects immediately after diagnosis, before treatment and after a given time of treatment[1]. It was proposed that studies of the rate of incorporation in relation to the time of incubation could elucidate the effect of these agents. The purpose of this paper is to describe such time-incorporation studies and further to give some data on the rate of incorporation of CH_3COONa-l-C^{14} into neutral fat and phospholipids as a function of time of incubation.

Methods and Materials

Blood was taken with A.C.D. solution and the leukocytes separated with polyvinyl pyrollidone solution as previously described (2). Separated leukocyte counts were performed in duplicate samples and the leukocyte suspension diluted with the subjects' own plasma in order that each sample should contain an equal concentration of leucocytes. To each leukocyte plasma mixture[1] CH_3COONa-l-C^{14} (spec. act. 48.9 mc/mg) 0.5 µc/ml, penicillin (100 units/ml) and streptomycin (100 µg/ml) were added. The mixture was then gently shaken at 37°C. in an atmosphere of 5% CO_2 and 95% O_2. Samples from each incubation mixture were withdrawn at standard time intervals and extracted. Extractions were performed by the method of Folch and co-workers (3). The lower phase containing the lipids was washed 4 times with the upper phase solution. After extraction, not more than 0.01% of the added radioactivity was found in the extract in the added form. The extract was evaporated to dryness under reduced pressure and taken up with petroleum ether (b. p. 60 — 70°C). Siliconized and sterilized glassware was

[1] Data for the concentration of leukocytes in the mixtures are given in the captions to each figure.

used throughout the separation and incubation process. The radioactivity of a small part of this material was measured by the direct technique of Entenman and co-workers (4) in a Geiger-Müller flow counter with an efficiency of 14% for C^{14} and a background of 14 c/min. No self absorption corrections were necessary, since the amounts of material applied to the planchets were extremely small. From four duplicate radioactivity measurements derived from four duplicate incubations each the percentage deviation was calculated. It had a range of ± 4.3 — 12.5% with a mean of ± 7.3%.

The remaining solution of total extracted lipids was separated by silicic acid chromatography into six fractions: a) hydrocarbons and cholesterol esters (H. C. + C. E.); b) triglycerides and free fatty acids (T. G. + F. F. A.); c) cholesterol (C); d) diglycerides (D. G.); e) monoglycerides (M. G.) and f) phospholipids (P. L.), according to the method of Fillerup and Mead (5) as modified by us and described elsewhere (2). The activity of the five first fractions was combined into one, referred to as neutral fat (N. F.). The radioactivity of this combined fraction was mainly due to the radioactivity of mono-, di- and triglycerides.

Results

Figure 1 shows the rate of incorporation of CH_3COONa-l-C^{14} into mixed lipids of leukocytes and plasma of the blood of 2 normal subjects (I, II), 1 leukaemic subjects without treatment (III) and 2 leukaemic subjects after treatment (IV, V). The type of leukaemia and the time and kind of treatment are described in the footnotes.

It can be seen that leukocytes from leukaemia subjects, treated with antimetabolites (IV) or alkylating agents (V) have a lower rate of incorporation, whereas leukaemic leukocytes derived from a non-treated patient (III) show a rate of incorporation similar to the normal ones (I and II). Furthermore, the apparent difference in case IV seems to be due to a more rapid levelling off of the incorporation curve; after 4 hours there was no further incorporation of CH_3COONa-l-C^{14} into lipids. In the case of the normal subjects (I, II) and the non-treated leukaemic patient (III) after an initial time lag, incorporation was almost linear for more than 8 hours. These incubations were performed on a mixture of separated leukocytes of nearly equal leukocyte concentration.

It is known (6) that the manipulation involved in the separation procedure reduces the rate of incorporation; therefore leukocytes from treated subjects might be more affected than those from untreated or normal ones. Experiments were therefore performed in which whole blood from treated patients was incubated and leukocytes were separated afterwards. Fig. 2 shows the results of such an experiment on blood of a patient with chronic lymphocytic leukaemia (C. L. L.). It can be seen that initial incorporation lasts for about 1 hour, after which there is almost no further incorporation. It seems likely that under these conditions the incorporation process differs from the normal one. It must be noted that the time of incorporation in this case was much shorter than in case IV.

This can be due to the increased concentration of leukocytes of this case. For this reason, the CH_3COONa-l-C^{14} concentration was increased to 1 μc/ml.

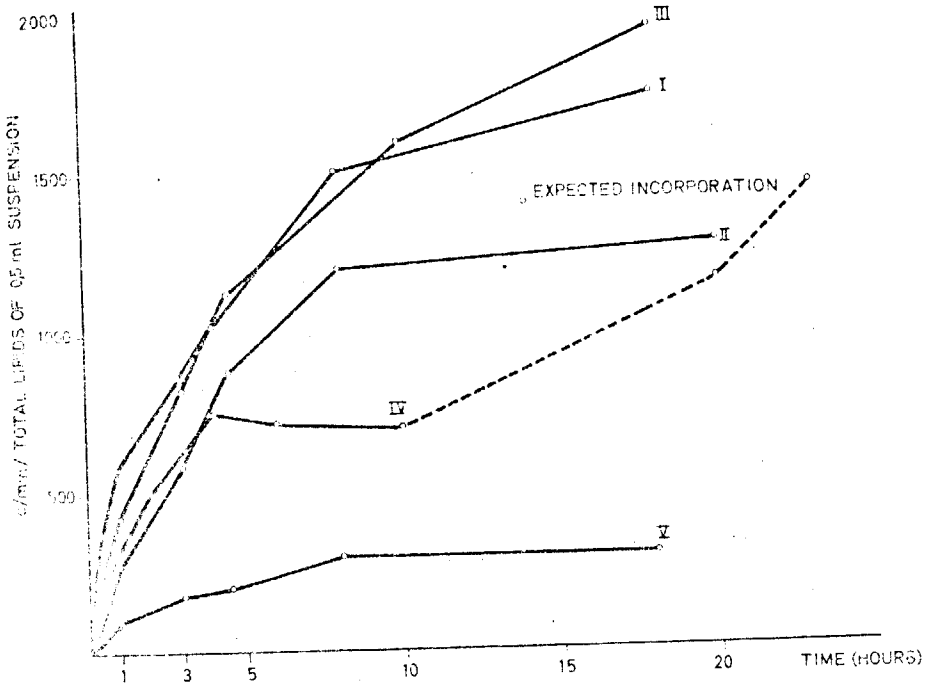


Fig. 1: Incorporation of radioactivity into mixed lipids of leukocytes and plasma. Incubation of mixtures of separated leukocytes with their own plasma in a concentration of about 8.5 × 10⁹ cells/mm³ and CH_3COONa-l-C^{14} 0.5 μc/ml. I, II: normal subjects; III: chronic myelogenic leukaemia (C. M. L.), untreated, W. B. C. 6300/mm³; IV: chronic myelogenic leukaemia (C. M. L.), treated with 400 mg 6-Mercaptopurine during the last 4 days before the experiment, W. B. C. = 6000/mm³. ----- Incorporation after further addition of CH_3COONa-l-C^{14}. Expected incorporation = Value of expected incorporation at the corresponding time after further addition of CH_3COONa-l-C^{14} to the incubation mixture of case IV calculated from the same experiment. V: chronic lymphocytic leukaemia (C. L. L.), treated with chlorambucil at intervals during the last year. He received 12 mg during the last 3 days before incubation, W. B. C. 18 700/mm³.

Fig. 2 also shows the incorporation into plasma lipids, due to exchange between the lipids of cells and plasma (7). This exchange seems to depend largely on the ratio of the cell lipids to plasma lipids. It is known (6) that more than

50% of the total incorporated radioactivity appears in the plasma lipids after 4 hours' incubation of whole blood. In the present case this fraction was 15%, probably due to the high ratio of cell lipids to plasma lipids. The 4-hour plasma lipid specific radioactivity data of this case are similar to those found by Marks and co-workers (6) after a 4-hour incubation of whole blood from a normal subject. (This is, of course, true only as far as percentage distribution of specific activities between plasma and cells is concerned).

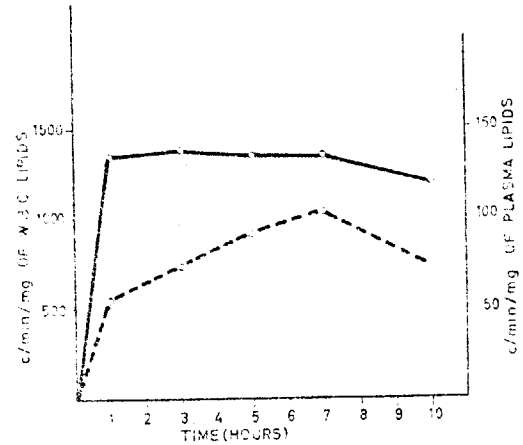
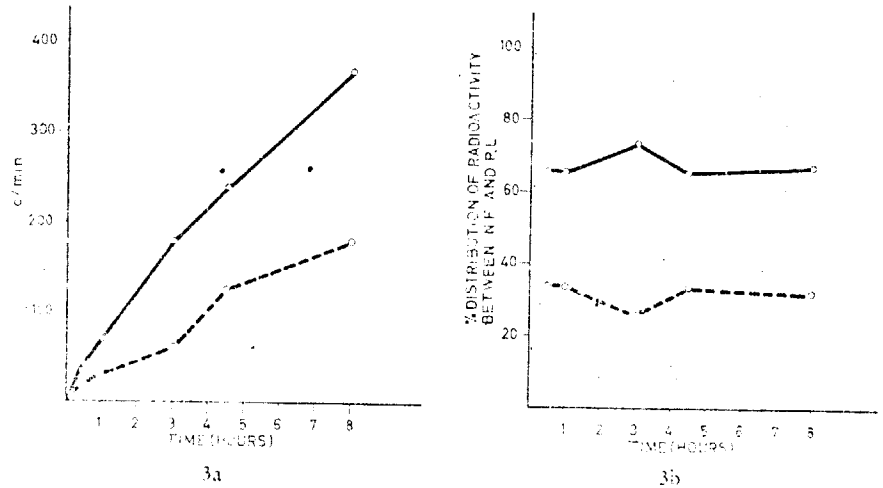


Fig. 2: Incorporation of CH_3COONa-l-C^{14} into mixed lipids of leukocytes and plasma. Incubation of whole blood from a C. L. L. patient with 402 600 W. B. C./mm³ and 98% lymphocytes. He received 140 mg of chlorambucil during the last 14 days. He had been previously treated at intervals with TEM. No alteration in the differential count was observed during or after treatment. Concentration of W. B. C. during incubation was 1.6 × 10⁹ cells/mm³ and CH_3COONa-l-C^{14} 1 μc/ml. ——— (W. B. C. lipid spec. radioactivity), — — — (plasma lipid spec. radioactivity).

Further experiments were performed to study the incorporation of CH_3COONa-l-C^{14} into neutral fat and phospholipid fractions as a function of time of incubation. Fig. 3a and Fig. 4a show representative results from a normal and leukaemic blood respectively. It can be seen from Fig. 3a (subject II of Fig. 1) that the rate of incorporation into phospholipids deviates more from linearity than the incorporation rate into neutral fats which is almost linear. Fig. 4a shows the results of similar studies on a patient with chronic lymphocytic leukaemia (C. L. L., same patient as in Fig. 2). It can be seen that the shape of the curves are similar to that of total lipid radioactivity incorporation of Fig. 2. When percentage distribution of radioactivity between N. F. and P. L. was plotted

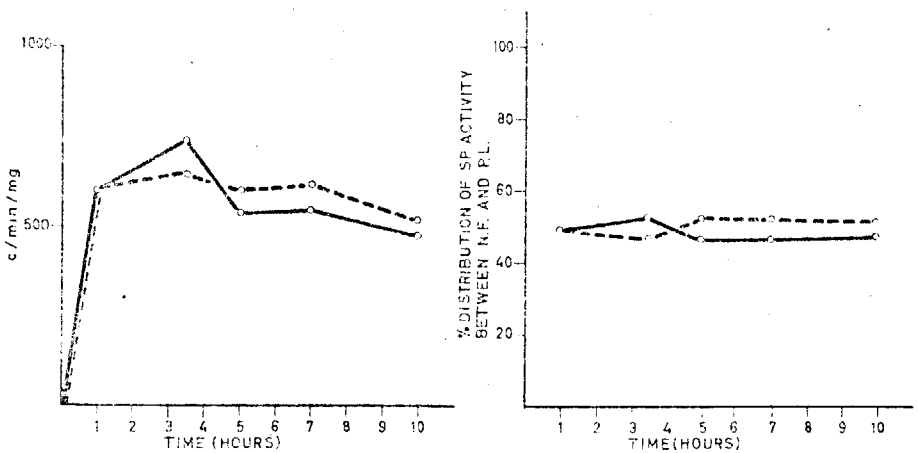
against time of incubation, two almost straight lines parallel to the axis of time were observed in both cases (Fig. 3b, Fig. 4b).



3a 3b

Fig. 3a: Incorporation of radioactivity into both plasma and W.B.C. neutral fats (N.F.) and phospholipids (P.L.) of blood from a normal subject

Fig. 3b: Percentage distribution of radioactivity between N.F. and P.L. ——— (N.F.) — — — (P.L.)



4a 4b

Fig. 4a: Effect of time of incubation on specific activity incorporation into W.B.C. neutral fat (N.F.) and phospholipids (P.L.) of a C.L.L.

Fig. 4b: Effect of time of incubation on percentage distribution of specific activity between N.F. and P.L. [illegible] (P.L.)

Discussion

The main purpose of the present work was to study whether cytostatic agents have a definite and direct effect on the lipid synthesis of leukaemic leukocytes, when the study of this synthesis is based on a CH_3COONa-l-C^{14} incorporation into total and fractionated lipids. The reduced incorporation after treatment with alkylating drugs or antimetabolites observed in a previous study (1) could have been due either to a reduced rate of incorporation throughout the incubation period or to an initially normal rate of incorporation with subsequent levelling off of the incorporation curve. The results of the present study suggest that the first assumption is not valid, at least for cases IV of Fig. 1 and C. L. L. of Fig. 2. In these cases, after an initial linear incorporation for 4 hours and 1 hour respectively, no further incorporation took place. It even seems that a decrease of the labelled lipids occurs later.

Treatment with alkylating agents or antimetabolites does not appear to affect uniformly all steps of the lipid synthesis in vitro, otherwise no initial rapid linear incorporation would take place and all curves would have a shape similar to the curve No. V of Fig. 1.

In case IV of Fig. 1, CH_3COONa-l-C^{14} was again added to the incubation mixture after 10 hours of incubation at which time none of the original CH_3COONa-l-C^{14} would remain in the reacting mixture. The newly added CH_3COONa-l-C^{14} seems in this case to have been again incorporated but at a markedly slower rate. It can be seen that the expected value of incorporation according to the quantity of CH_3COONa-l-C^{14} added[1], was reached after a longer time than that found when the radioactive material was added before incubation.

It seems likely therefore that treatment affects such lipid synthesis in an indirect way. Consequently experiments performed with more specialized precursors such as fatty acids, in conjunction with studies of the effect of cytostatic agents in vitro, might shed more light on the effect of such agents on the rate of fatty acid incorporation.

Furthermore, a study of the time activity curves in Figs. 3a and 4a gives no definite evidence that neutral fat and phospholipids of leukocytes have any precursor-product relation in vitro.

On the other hand, alkylating agents seem to affect the synthesis of neutral fat and phospholipids in the same way.

[1] It was supposed that the incubation mixture did not at that time contain available CH_3COONa-l-C^{14}.

Summary

1. Separated leukocytes or total blood from normal subjects, untreated leukaemic patients and from leukaemic patients treated with cytostatic agents were incubated with CH_3COONa-l-C^{14}. Radioactivity of mixed lipids was measured at standard time intervals.

2. The time incorporation curve observed with leukocytes from treated leukaemic patients showed after an initial linear part, a more rapid levelling off than the curves observed with leukocytes from untreated and normal subjects.

3. Therefore, an indirect effect of treatment on leukocyte lipid synthesis seems to be present.

4. Phospholipid and neutral lipid synthesis by leukaemic leukocytes was also studied. The results give no evidence that these fractions as a whole have any precursor-product relation.

Résumé

1. Des leucocytes séparés ou du sang des personnes normales, de malades leucémiques non traités et traités par zytostatiques ont été incubés avec CH_3COONa-l-C^{14}. La radioactivité des lipides mixtes a été déterminée en intervalles réguliers.

2. La courbe, fixation — temps de globules blancs des leucémiques traités montrait après une partie précoce linéaire un aplatissement plus rapide que les courbes des globules des leucémiques non traités et personnes normales.

3. On adopte un effet indirecte de la thérapie sur la synthèse des lipides des leucocytes.

4. La synthèse des phospholipides et lipides neutres par des leucocytes leucémiques a été examiné également. Les résultats ne donnent aucune évidence d'une correlation produit-précurseur dans cette fraction sommaire.

Zusammenfassung

1. Abgetrennte Leukozyten oder Gesamtblut von Normalpersonen, unbehandelten Leukämiepatienten und von Leukämiepatienten, die mit Zytostatika behandelt worden waren, wurden mit CH_3COONa-l-C^{14} inkubiert. Die Radioaktivität der gemischten Lipoide wurde in regelmäßigen Zeitabständen gemessen.

2. Die Zeit-Einbaukurve, die bei Leukozyten von behandelten Leukämiepatienten beobachtet wurde, zeigte nach einem zunächst linearen Teil ein schnelleres Abflachen als die Kurven, die bei Leukozyten von Leukämiepatienten und von Normalpersonen gefunden wurden.

3. Ein indirekter Effekt der Therapie auf die Lipoidsynthese der Leukozyten scheint daher vorhanden zu sein.

4. Die Phospholipoid- und Neutrallipoidsynthese durch leukämische Leukozyten wurden ebenfalls untersucht. Die Ergebnisse liefern keinen Beweis, daß diesen Fraktionen als Ganzes irgendeine Vorläufer-Produkt-Beziehung best[illegible].

References

(1) Miras, C. J., and J. D. Mantzos: Studies on the Metabolism of Lipid by Leuka[illegible] Leukocytes before and after Treatment (in vitro). Proc. of the Greek Academy of Sci[illegible] 36: 26 (1961).

(2) Malamos, B., C. Miras, G. Lewis and J. Mantzos: The in vitro Incorpora[illegible] of Acetate-l-C^{14} into Normal and Leukaemic Leukocyte Lipids. J. Lipid Research (u[illegible] publication).

(3) Folch, J., M. Lees and G. H. Sloane-Stanley: A Simple Method for the Isola[illegible] of Total Lipids from Animal Tissues. J. Biol. Chem. 226: 497 (1957).

(4) Entenman, C. S. R. Lerner, J. L. Chaikoff and W. C. Dauben: Determination of Carbon-14 in Fatty Acids by Direct Mount Technic. Prof. Soc. Exp. Biol. Med. 70: 364 (1949).

(5) Fillerup, D. L., and J. F. Mead: Chromatographic Separation of the Plasma Lipids. Proc. Soc. Exp. Biol. Med. 83: 574 (1953). Plasma Lipids in Fat Deficiency. ibid 86: 449 (1954).

(6) Marks, P. A., A. Gellhan and Ch. Kidson: Lipid Synthesis in Human Leukocytes, Platelets, and Erythrocytes. J. Biol. Chem. 235: 2579 (1960).

(7) Lovelock, J. E., A. T. James and C. E. Rowe: The Lipids of Whole Blood. Biochem. J. 74: 137 (1960).

Local effects of various anions and H^+ on dog limb and coronary vascular resistances

Am. J. Physiol. 203: 125-32, (1962)

J. I. MOLNAR, J. B. SCOTT, E. D. FROHLICH, AND F. J. HADDY (With the Assistance of Judith N. Huizenga, Booker T. Swindall, and Sim Swindall)
Veterans Administration Research Hospital and Northwestern University Medical School, Chicago, Illinois; U. S. Army Medical Research Laboratory, Fort Knox, Kentucky; and University of Oklahoma Medical Center, Oklahoma City, Oklahoma

MOLNAR, J. I., J. B. SCOTT, E. D. FROHLICH, AND F. J. HADDY. *Local effects of various anions and H^+ on dog limb and coronary vascular resistances.* Am. J. Physiol. 203(1): 125-132, 1962. The effect of intra-arterial infusion of an isosmotic solution of various sodium salts and acids on pressure gradients in the forelimb and heart was studied during constant rate blood perfusion. Forelimb resistance was reduced by Na pyruvate, Na acetate, Na nitrite, and Na citrate, by hydrochloric, nitric, lactic, pyruvic, acetic, and citric acids, and unchanged by NaCl, $NaHCO_3$, $NaNO_3$, and Na lactate. The molecular activity of Na acetate or Na nitrite exceeded that of Na pyruvate. All changes resulted mainly from fall of small vessel resistance. Na acetate also increased venous resistance, probably by a remote mechanism, and limb weight; the latter was more pronounced in the absence of artificial blood perfusion. Coronary resistance was decreased by Na acetate, Na nitrite, and Na citrate, increased slightly by $NaHCO_3$, and unchanged by NaCl and Na lactate. Hence, the local effect of slight excess blood pyruvate, acetate, nitrite, citrate, or hydrogen is active arteriolar dilation, the activity of acetate or nitrite exceeds that of pyruvate, and acetate greatly increases tissue water storage.

AMONG THE MANY REASONS for an interest in the local vascular actions of the hydrogen ion and natural occurring anions is the possibility that these substances participate in local regulation of blood flow. Arterioles systematically dilate following arterial occlusion, reduction of perfusion pressure, or increased muscular activity. Among the possible causes of the dilation are decrease of transmural pressure, decrease of oxygen tension, and accumulation of vasoactive metabolites. Though there have been a number of studies on the local vascular actions of transmural pressure and oxygen, there has been no systematic study of the absolute and relative actions of the hydrogen ion and of a series of anions on the resistances to blood flow through intact small and large blood vessels. Hence, the possible importance of their role in local regulation of blood flow is difficult to evaluate. This paper presents a systematic study in the dog forelimb and coronary vascular beds.

The events leading to the study were as follows. While studying the action of the magnesium ion, it was noted that forelimb resistance decreased more during intrabrachial infusion of an isotonic solution of magnesium acetate than during intrabrachial infusion of an isotonic solution of magnesium chloride or magnesium sulfate, even though the delivery rate of magnesium ion was the same during each infusion (1). This led to a comparison of isotonic sodium acetate and isotonic sodium chloride; it was found that the former solution decreased resistance greatly, whereas the latter solution, delivered at the same volume rate, was almost without effect (1). Further, sodium acetate sometimes simultaneously increased venous resistance (1). These findings indicated unusual activity of the acetate ion and stimulated a more detailed study of acetate, as well as a search for other active anions. We have previously reported that, relative to a series of cations, the chloride, sulfate, and biphosphate ions seem to be without effect on intact blood vessels (1-3).

METHODS

The local effects of various organic and inorganic sodium salts and acids on resistance to blood flow through the forelimb and coronary vascular beds were compared in the anesthetized dog. This was accomplished by perfusing the beds with arterial blood at constant rates and observing the effects of intrabrachial and intracoronary infusion of isosmotic solutions of the salts and acids on pressure gradients. Infusion rate was sufficient to increase anion or hydrogen ion concentration in the perfused bed, but insufficient to greatly affect concentrations in the general circulation.

Received for publication 22 January 1962.

Forelimb vascular bed. Forty-nine dogs weighing 13.6–18.2 kg were anesthetized with sodium pentobarbital, 30 mg/kg. The brachial artery, third superficial volar metacarpal artery, and second superficial dorsal metacarpal vein of the right forelimb, and the femoral artery of the right hindlimb were exposed through small incisions. Heparin sodium, 4 mg/kg, was injected intravenously. The femoral and brachial arteries were ligated and a blood pump (Sigmamotor pump, model T-6SH, Sigmamotor, Inc., Middleport, N.Y., modified to deliver a constant flow despite change in input or output pressure over the range 0–300 mm Hg) was interposed between the proximal segment of the femoral artery and the distal segment of the brachial artery. Forelimb flow rate was set at 100 ml/min. A 22-gauge needle was inserted cephalad into the right cephalic vein through the intact skin, a 0.5-mm-diameter glass tube was manipulated distally in the ligated metacarpal vein until the tip lay in the toe web, a 0.5-mm glass tube was inserted 1 cm distally into the ligated metacarpal artery so that the tip lay at the upper edge of the footpad, a 22-gauge needle was inserted into the pump perfusion tubing just proximal to the tubing's entrance into the brachial artery, and a polyethylene tube was manipulated up the proximal segment of the brachial artery into the aorta. All were attached, through a multiple stopcock arrangement, to one pressure transducer having a volume displacement of 0.01 mm³/100 mm Hg (resistance wire pressure transducer, model P-23 Gb, Statham Laboratories, Inc., Los Angeles). Pressures were sequentially recorded on a direct-writing oscillograph. The tubes and needles were periodically flushed with heparinized saline from a pressurized reservoir. The test solutions were infused into the perfusion circuit between the femoral artery and pump. Hence, the infusions did not alter flow rate through the limb. In selected experiments, blood was simultaneously withdrawn from the brachial artery and cephalic vein before, during, and after infusion of a test solution and blood pH and plasma potassium concentrations determined by standard methods. In others, limb weight was recorded by a previously described technique (4).

TABLE 1. *Nature of test solutions infused into dog forelimb*

	%	pH	mOs/liter	mEq/min*
NaCl	0.90	5.84	295	0.90
HCl	0.92	0.82	293	1.62
$NaHCO_3$	1.41	8.59	295	1.68
$NaNO_3$	1.57	6.10	293	1.03
HNO_3	1.03	0.80	290	1.04
Na lactate	1.87	6.75	312	1.67
Lactic acid	2.46	2.07	292	1.55
Na pyruvate	1.70	5.72	292	0.99
Pyruvic acid	2.42	1.52	303	1.76
Na acetate	1.25	8.02	268	0.97
Acetic acid	1.90	2.59	317	2.02
$NaNO_2$	1.12	6.50	303	1.04
Na citrate $2H_2O$	3.05	7.45	386	1.01
Citric acid	5.52	1.52	310	5.59

* At an infusion rate of 0.1 ml/min.

TABLE 2. *Effects of intrabrachial infusion (6.4 ml/min) of 3 NaCl solutions, of slightly different osmolal concentrations, on vascular pressures (mm Hg) in forelimb perfused with 100 ml blood/min**

	C	290 mOs/liter†	C‡	C	321 mOs/liter†	C‡	C	375 mOs/liter†	C‡
Aorta	112	113	115	113	113	113	114	116	116
Brachial artery	103	99	105	106	104	111	112	106	117
Small artery	69	65	73	74	66	78	78	72	82
Small vein	9	10	10	10	10	10	10	10	10
Cephalic vein	6	7	7	7	7	7	7	7	7

* Avg. of 7 exp. † 1 min after onset of infusion. ‡ 5 min after stopping infusion.

Effects upon pressures and weight in the presence of natural flow were studied in six additional dogs. The blood pump was not utilized. Intrabrachial infusion was accomplished through a small side branch. A second side branch was utilized to measure brachial arterial pressure. The other limb pressures were measured as described above.

Coronary vascular bed. Twenty-six dogs weighing 10.7–18.0 kg were anesthetized with sodium pentobarbital, 30 mg/kg. The coronary preparation has been described in detail in previous communications (5, 6). In brief, the heart was exposed through the right fourth intercostal space, heparin injected intravenously (5 mg/kg) and plastic cannulas introduced into the superior and inferior vena cavae via the right atrium. A rotating disc oxygenator, a blood heater, and a blood pump were interposed between these cannulas and the left femoral artery, and the body perfused at 1240–1540 ml/min. Body temperature was maintained at 37° C.

The coronary vascular bed was perfused with a second pressure independent pump interposed between the right femoral artery and the ascending aorta. The aorta and pulmonary artery were cross clamped 3 cm from the heart, thus diverting the output of the pump through the entire coronary vascular bed. Flow rate was adjusted to a value between 50 and 125 ml/min. In order to collect coronary venous blood from the right heart, a cannula having multiple holes was introduced into the right atrium and threaded through the tricuspid valve in such a way as to render the valve incompetent. This blood was returned to the venous limb of the systemic perfusion circuit. Left heart blood (from arteriolaminal, Thebesian, and bronchial vessels) was collected with another catheter inserted into the left atrium and ventricle through a superior pulmonary vein. A resistance wire pressure transducer was utilized to measure pressure in the coronary perfusion tubing just prior to entrance into the ascending aorta.

Solutions of NaCl (330 mOs/liter), $NaHCO_3$ (310 mOs/liter), Na lactate (329 mOs/liter), Na citrate

TABLE 3. *Average effects* of active salts on total and segmental pressure gradients (mm Hg) in dog forelimb perfused with 100 ml blood/min*

	Na Pyruvate	Na Acetate	$NaNO_2$	Na Citrate
Total	−19	−53	−56	−23
Arterial	+4	+1	−3	+9
Small vessel	−24	−57	−51	−33
Venous	+0.4	+2.1	−1.2	0.0

* Change from control value 1 min after starting infusion of isosmotic solution into brachial artery at 6.4 ml/min in 9 animals.

(314 mOs/liter), Na acetate (303 mOs/liter), and $NaNO_2$ (315 mOs/liter) were infused into the coronary perfusion circuit between the pump and the coronary vascular bed at rates of 0.2, 0.5, 0.8, 1.2, 1.6, 2.1, and 2.6 ml/min, in that order. Each infusion rate was maintained for 30 sec. Perfusion pressure and electrocardiogram were recorded continuously; pressure, heart rate, and QT interval were measured at the end of each 30-sec period.

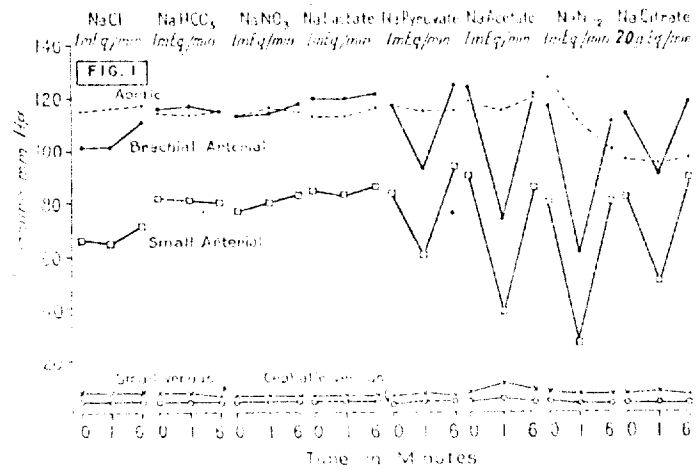
RESULTS

Forelimb. Due to practical difficulties in preparation, the test solutions varied slightly in osmolal concentration (Table 1). We have previously shown that greatly hypertonic or hypotonic solutions affect resistance to flow through this preparation (1). In order to determine the importance of slight differences in osmolality, three solutions of sodium chloride, having osmolal concentrations of 266, 294, and 325 mOs/liter, respectively, were infused into the circuit at 6.4 ml/min (the same rate utilized for the definitive study of the test solutions). Average arterial pressures decreased slightly during infusion of each solution, but the magnitudes of the changes were essentially the same for each of the three solutions (Table 2). These changes probably result from decrease in blood viscosity due to dilution of the blood by the infusate (1).

In 14 preliminary experiments, it was found that brachial and small arterial pressures were essentially unaffected by the solutions of NaCl, $NaHCO_3$, $NaNO_3$, and Na lactate, but decreased as a function of the infusion rate of each of the other test solutions. With these latter solutions, the onset of action was almost immediate; the pressures were in a steady state within 1 min of starting the infusion; and, in most instances, the pressures returned to the control value within 5 min of stopping the infusion. An infusion rate of 6.4 ml/min (approx. 1 mEq/min for each salt except Na citrate) always produced a measurable limb response, though the magnitude of the response to a given solution varied from animal to animal. The limb response was unaccompanied by large changes in aortic pressure except perhaps in the cases of $NaNO_2$ and citric acid. Citric acid sometimes produced limb tetany and variations in respiratory pattern. The sequence of administration of the test solutions did not alter the responses.

In view of these preliminary observations, it was decided to infuse all 14 solutions into the same group of animals at 6.4 ml/min for 1 min, in the order presented in Table 1, and to record pressures immediately before infusion, 1 min after onset of infusion, and 5 min following discontinuance of infusion. It was felt that this would provide a basis for comparison of the relative activities of the anions. All the anions except citrate are monovalent. Hence, except for citrate, approximately the same number of sodium and anion particles are delivered per unit time during each infusion. Differences in responses may therefore be attributed to differences in anions.

Figure 1 presents average effects of the eight sodium salts in the same nine animals. Brachial and small arterial pressures fell almost immediately following the



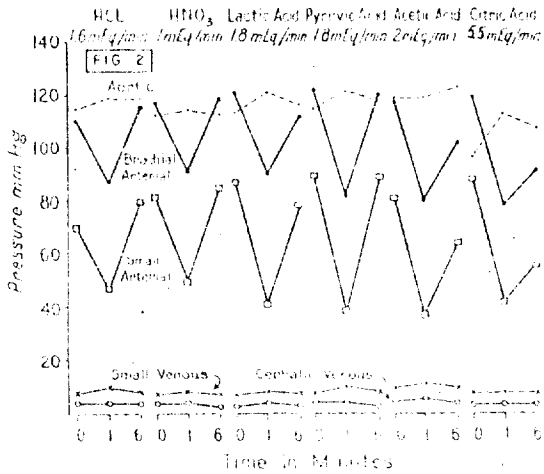


FIG. 1. Effects of 8 different sodium salts on forelimb vascular and aortic pressures with rate of blood flow in brachial artery held constant at 100 ml/min. All solutions isosmotic to plasma and infused into brachial artery at 6.4 ml/min. Avg. of 9 exp.

FIG. 2. Effects of 6 different acids on forelimb vascular and aortic pressures with rate of blood flow held constant at 100 ml/min. All solutions isosmotic to plasma and infused into brachial artery at 6.4 ml/min. Avg. of 9 exp. with same animals as presented in Fig. 1.

start of each infusion of Na pyruvate, Na acetate, $NaNO_2$, or Na citrate. Small venous pressure fell during each infusion of $NaNO_2$ and rose during seven of nine infusions of Na acetate. Cephalic venous pressure fell slightly during four of nine infusions of $NaNO_2$ and rose during four of nine infusions of Na acetate. $NaNO_2$ decreased aortic pressure in all cases and this pressure did not return to the control value during the 5-min period following infusion. None of the pressures were regularly affected by NaCl, $NaHCO_3$, $NaNO_3$, and Na lactate.

Table 3 presents magnitudes of changes in total and segmental pressure gradients during infusion of the four active salts. Each solution always decreased total resistance mainly by decreasing the resistance to flow through small vessels. These resistances fell more during infusion of Na acetate and $NaNO_2$ than during infusion of Na pyruvate in each of the nine experiments. Na citrate should not be compared, since it was infused at a different milliequivalent and molecular rate. It will be noted, however, that the magnitude of the response to 2.0 mEq Na citrate/min was similar to the response to 1.0 mEq Na pyruvate/min and about one-half the response to 1.0 mEq Na acetate or $NaNO_2$/min, and that 2.0 mEq Na citrate/min supplies fewer molecules than 1.0 mEq monovalent salt/min. $NaNO_2$ decreased resistance to flow through veins in nine of nine experiments. Na acetate increased resistance to flow through veins in five of nine experiments. Resistance to blood flow through arteries was affected irregularly. Na pyruvate, Na acetate, $NaNO_2$, and Na citrate decreased arterial resistance in three, four, five, and one experiments, respectively, increased arterial resistance in six, four, two, and seven experiments, respectively, and had no effect in zero, one, two, and one experiments, respectively.

Figure 2 presents average effects of the six acids in the same nine animals. The volume infusion rate of 6.4 ml/min delivered 1 mEq/min only in the case of HNO_3. Each acid immediately lowered brachial and small arterial pressures in each of the nine experiments. Each acid raised small venous pressure in one or more experiments (3, 3, 2, 1, 6, and 1 exp. during infusion of HCl, HNO_3, lactic acid, pyruvic acid, acetic acid, and citric acid, respectively). All acids, except citric, elevated cephalic venous pressure in one or more experiments (1, 1, 1, 1, and 3 exp. during infusion of HCl, HNO_3, lactic acid, pyruvic acid, and acetic acid, respectively). The actions of lactic acid, acetic acid, and citric acid persisted longer than those of the other acids. Citric acid produced tetanic-like movements of the limb in five experiments and variations in the respiratory pattern in six experiments. Lactic acid, pyruvic acid, and citric acid regularly increased aortic pressure. The increase was greatest and most persistent in the case of citric acid.

TABLE 3. *Average effects[a] of salts on total and segmental pressure gradients (mm Hg) in dog forelimb perfused with 100 ml blood/min*

	NaCl	$NaHCO_3$	$NaNO_2$	Na acetate	Na citrate
Total	−23	−27	−33	−41	−40
Arterial	+5	+5	+14	+11	+16
Small vessel	−30	−33	−47	−53	−46
Venous	+1.1	+0.8	+0.4	+1.2	+0.9	+0.4

[a] Change from control value 1 min after starting infusion of isosmotic solution into brachial artery at 6.4 ml/min in 9 animals.

TABLE 4. *Average effects[a] of acids on total and segmental pressure gradients (mm Hg) in dog forelimb perfused with 100 ml blood/min*

	HCl	HNO_3	Lactic Acid	Pyruvic Acid	Acetic Acid	Citric Acid
Total	−23	−27	−33	−41	−40	−40
Arterial	+5	+5	+14	+11	+5	+16
Small vessel	−30	−33	−47	−53	−46	−49
Venous	+1.1	+0.8	+0.4	+1.2	+0.9	+0.4

[a] Change from control value 1 min after starting infusion of isosmotic solution into brachial artery at 6.4 ml/min in 9 animals.

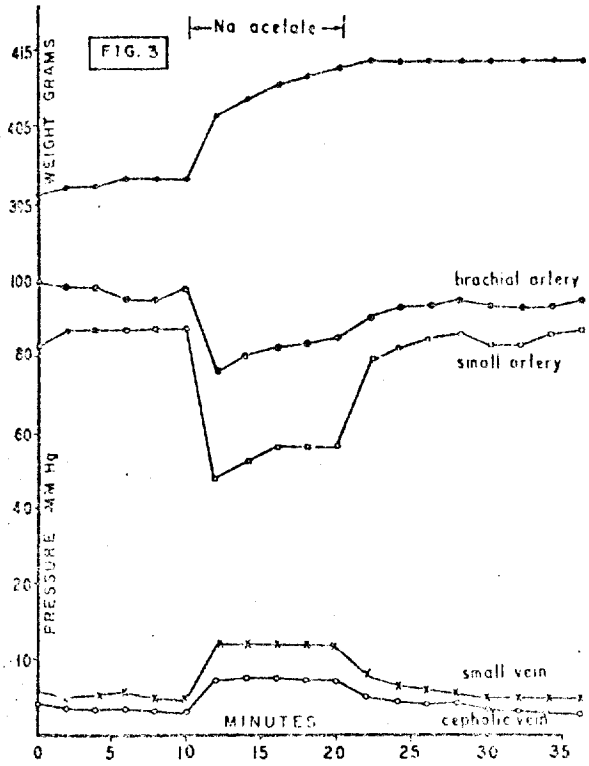
TABLE 5. *Average effects of salts and acids on brachial arterial and cephalic venous blood pH and plasma potassium concentration in dog forelimb perfused with 100 ml blood/min*

	pH		Potassium	
	A	V	A mEq/liter	V mEq/liter
Control	7.30 (10)	7.38 (10)[b]	3.37 (6)	3.47 (6)
Salt[a]	7.34 (11)[b]	7.41 (10)	3.11 (6)	3.15 (6)
Control[b]	7.38 (11)[b]	7.41 (10)[b]	3.52 (6)	3.38[b] (6)[b]
Control	7.36 (8)	7.30 (8)	3.47 (6)	3.39 (6)
Acid[a]	6.72 (8)[b]	6.85 (6)	3.53 (6)	3.40 (6)
Control[b]	7.35 (8)[b]	7.26 (7)	3.45 (6)	3.65 (6)

No. of exp. in parentheses. [a] 1 min after start of infusion at 6.4 ml/min into brachial artery. [b] 5 min after stopping infusion.

Table 4 presents the magnitude of change in total and segmental pressure gradients from control values. Each acid decreased total resistance by decreasing the resistance to flow through small vessels. On the average, each acid decreased small vessel resistance more than total resistance and each acid increased arterial resistance.

The pH of brachial arterial and cephalic venous blood was determined before, during, and after one or more 6.4 ml/min infusions of each salt (NaCl, $NaNO_2$, and $NaHCO_3$ studied twice); pH was not measurably affected in any case. The concentration of potassium in brachial arterial and cephalic venous blood was measured before, during, and after 6.4 ml/min infusions of $NaNO_2$ (twice), Na acetate, Na citrate, Na pyruvate, and Na lactate. Potassium concentration in brachial arterial and cephalic venous plasma decreased about equally in all cases and by an amount predicted from dilution by the infusate. Limb blood pH was also studied during one or more 6.4 ml/min infusions of each acid (HCl and HNO_3 studied twice). Arterial and venous pH decreased about equally during each infusion. However, the magnitudes of the decreases varied from acid to acid, smaller changes being produced by HCl and HNO_3. Limb plasma potassium concentration was studied during 6.4 ml/min infusions of nitric acid (twice), acetic acid, pyruvic acid, citric acid, and lactic acid. Each acid except citric acid produced a slight increase in both the arterial and venous concen-



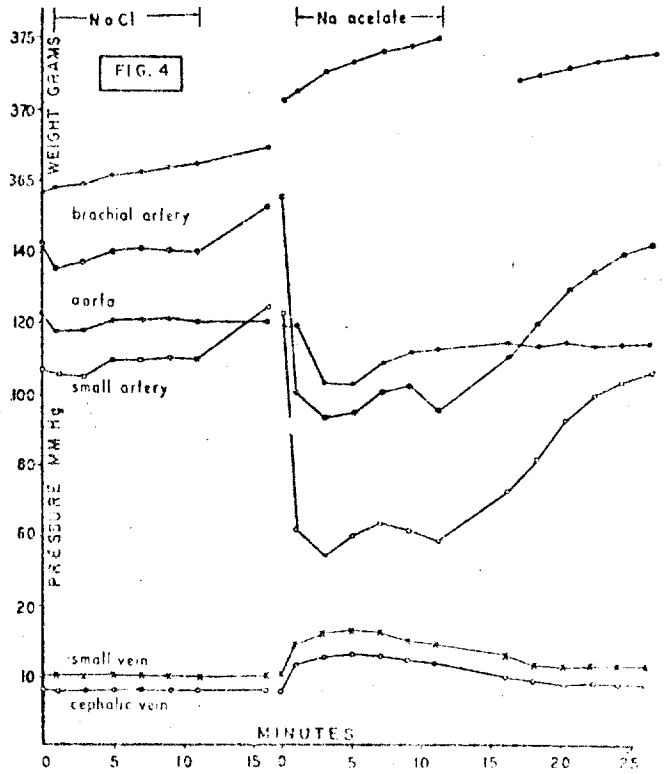


FIG. 3. Effects of a 30 ml/min intrabrachial infusion of isosmotic solution of Na acetate on vascular pressures and weight of naturally perfused forelimb.

FIG. 4. Effects of 6.8 ml/min intrabrachial infusions of isosmotic solutions of sodium chloride and sodium acetate on forelimb vascular and aortic pressures and limb weight, with rate of blood flow in brachial artery held constant. Avgs. of 13 animals. Blood flow was 50, 70, and 100 ml/min in 1, 7, and 5 exp., respectively. Only 12 animals included in wt. avg. during last 10-min period. Time interval only a few minutes between 1st and 2nd period.

trations of potassium. Average effects are presented in Table 5.

The observation that Na acetate frequently raises venous resistance at the same time that it decreases small vessel resistance suggested that this agent might greatly increase the store of tissue water. For this reason, the effect of Na acetate was studied in the naturally perfused limb. The blood pump was not utilized. Pressures and limb weight were recorded during infusion of Na acetate into the brachial artery at 3 ml/min for 10 min. In six animals, weight gain averaged 2.2 g during a 10-min control period [this preparation spontaneously [illegible] weight at a diminishing rate because of a slightly dependent position in relation to the heart (4–7), 7.8 g [illegible] infusion of Na acetate for 10 min, and 0.7 g [illegible] the period 5–15 min after stopping the infusion. [illegible] acetate decreased brachial and small arterial pressures [illegible] and 2.3 mm Hg, respectively, and raised small [illegible] cephalic venous pressures 6.0 and 2.1 mm Hg, respectively. Figure 3 presents a typical experiment. In [illegible] to single out the contribution of venous resistance [illegible] weight gain, flow was held constant and limb [illegible] compared during successive 10-min infusions of NaCl and Na acetate at 6.8 ml/min in 13 experiments. [illegible] of these experiments, the small quantity of [illegible] flow (7) was eliminated by placement of a tourniquet around all upper limb structures except the brachial artery, brachial vein, cephalic vein, and nerves of the brachial plexus. Na acetate increased average small and large vein pressures, the former more than the latter, as well as the average rate of weight gain (Fig. 4). The average gain was 1.7 g during infusion of NaCl, 3.7 g during infusion of Na acetate and 1.8 g during the period 10–15 min after stopping the infusion. Small and large venous pressures increased in ten of the thirteen experiments and in all seven in which the tourniquet was in place. The rate of weight gain was greater during Na acetate infusion than during NaCl infusion in eight of the ten experiments in which venous pressures rose, but not in the three experiments in which venous pressures were unaffected. Aortic pressure fell in 10 of 13 experiments but tended to recover, along with brachial and small arterial pressures, after the 5th min of infusion.

Coronary vascular resistance. Coronary perfusion pressure decreased as a function of the infusion rate of the solution of $NaNO_3$, Na acetate, or Na citrate in each experiment. The average effects of the three solutions were approximately equal (Fig. 5). However, at a given volume infusion rate, an isosmotic solution of Na citrate provides lower molecules than an isosmotic solution of $NaNO_3$ or Na acetate. Perfusion pressure increased

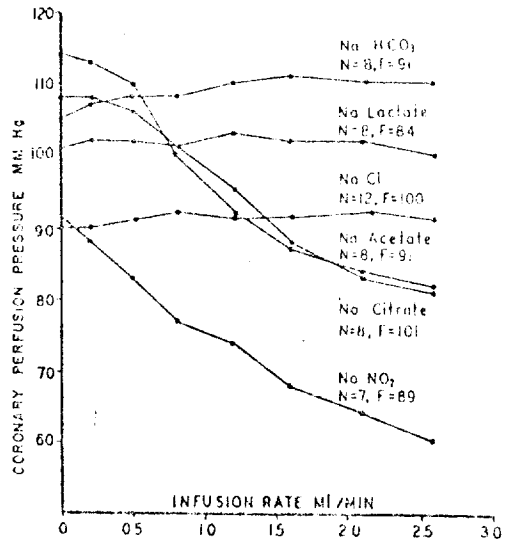


FIG. 5. Coronary perfusion pressure with rate of coronary blood flow constant as function of infusion rate of 6 different isosmotic solutions of sodium salts. Each infusion rate maintained for 30 sec, at which time pressures were steady. N refers to number of animals included in each average and F to average rate of blood flow in ml/min in each group. $NaHCO_3$, $NaNO_2$, and Na lactate were infused into one group of animals, Na acetate and Na citrate into a second group; NaCl into a third group.

slightly as a function of the infusion rate of the solution of $NaHCO_3$. The solutions of NaCl and Na lactate were without effect.

Heart rate decreased slightly during infusion of Na acetate and sodium citrate and QT interval increased slightly during infusion of sodium citrate. Neither of these parameters were affected by the other solutions (Table 6). Na acetate and Na citrate produced small and irregular changes in the proportion of time spent in electrical systole by the ventricle (calculated from QT interval × no. beats/min 60).

DISCUSSION

These studies show that slight local excess hydrogen, pyruvate, acetate, or citrate produces active arteriolar dilation. Therefore, if they accumulate during arterial occlusion, muscular activity, or reduction of perfusion pressure, they could participate in the production of the arteriolar dilation characteristic of these conditions. The study also shows that acetate and citrate are particularly active on the arteriole and that acetate constricts veins as well. Acetate, then, increases capillary hydrostatic pressure and water efflux from the capillary more than do the other ions. This suggests a role for acetate in tissue water storage.

The effects of sodium pyruvate, sodium acetate, and $NaNO_2$ result from the anions rather than from the sodium ion. Inasmuch as sodium concentrations in isosmotic solutions of these salts are similar to sodium concentration in plasma, the latter is not significantly affected by infusion of the solutions at any rate. On the other hand, since anion concentrations in these solutions are much higher than anion concentrations in plasma, the latter will be increased by any infusion rate. Hence, changes in resistance may be attributed to the anion rather than to the sodium ion and absence of a resistance change signifies absence of anion activity at the infusion rate studied. The magnitude of the increase in anion concentration during infusion at a given rate differs for each anion depending upon the concentrations of the anion in plasma and in the solution. Normal dog plasma anion concentrations in mEq/liter are approximately chloride 106, bicarbonate 20, nitrate 0.0, lactate 2.6, pyruvate 0.1, acetate 0.6, nitrite 0.0, and citrate 0.4. Assuming uniform distribution of infused anion in plasma and red cells, it may be calculated that infusion of the salt solutions utilized in these experiments at 6.4 ml/min on the proximal side of a pump delivering blood at 100 ml/min will increase the above concentrations in limb plasma as follows: chloride 3.0, bicarbonate 9.5, nitrate 10.3, lactate 10.4, pyruvate 9.9, acetate 9.7, nitrite 10.3, and citrate 19.9. It should be noted that calculated increments are similar for pyruvate, acetate, and nitrite and that resistance decreases more during infusion of acetate or nitrite than during infusion of pyruvate.

The absence of an acute local effect of sodium lactate has been demonstrated previously. Bellet et al. (3) found that arterial injection of as much as 12 ml/kg (isotonic) Na lactate failed to affect resistance to blood flow through the hind limbs of anesthetized dogs.

Pyruvate, acetate, nitrite, and citrate undoubtedly decrease resistance mainly through active arteriolar dilation. Total resistance decreases because of decrease of resistance to flow through vessels less than 0.5 mm in diameter. This decrease in small vessel resistance results from dilation rather than from reduction of blood viscosity because dilution by the infusate is insignificant (1) and because, at a given infusion rate, the magnitude of the resistance decrease varies from solution to solution. The dilation is active rather than passive because the dilation occurs despite fall of small vessel transmural pressures and because isosmotic solutions do not dehydrate vessel walls (1). The small vessels which dilate are mainly small arteries and or arterioles rather than capillaries and small veins because the magnitude of the decrease of small vessel resistance greatly exceeds the absolute value for resistance to flow through capillaries and small veins (9). The active small arteriolar dilation results from local actions of the infused anion because dilation is apparent within seconds of the onset of intra-arterial infusion. It is possible, however, that the local actions are partially altered by remote action by the end of 1 min of infusion. For example, if significant quantities of the infusate enter the systemic circulation, the blood pressure might fall because of widespread arteriolar dilation. The pressure fall would be sensed by pressoreceptors and result in a sympathetic adrenal discharge. The latter would partially antagonize

the local dilatory action of the anion. This seems especially likely in the case of nitrite.

Pyruvate, acetate, and nitrite also actively dilate larger arteries (0.5–5.0-mm diameter). This is clear because arterial resistance sometimes falls despite decrease of arterial intraluminal and, hence, transmural pressures. Arterial resistance does not change or rises in other experiments because passive constriction due to decrease of intraluminal pressure is equal to, or more pronounced than, active dilation due to a direct effect of the anion.

Nitrite also dilates veins. This, then, is the only anion studied which clearly dilates along the entire length of the bed. The acetate ion is particularly interesting because, like histamine (9), it simultaneously dilates arterioles and constricts veins. The venous constriction includes veins even larger than 0.5–5.0-mm diameter because cephalic venous pressure also rises. The venous constrictor action of intrabrachially-administered histamine results, at least in part, from a sympathico adrenal discharge subsequent to fall of systemic arterial pressure. This may also be the cause of acetate venous constriction. Aortic pressure falls during the 2nd min of infusion but partially recovers because of arteriolar constriction in subsequent minutes (Fig. 1). The fact that nitrite dilates veins despite fall of aortic pressure may simply indicate that the local action of nitrite on veins predominates over the remote action. Regardless of the mechanism of the acetate venous constriction, the constriction clearly increases the rate of water filtration into tissues. Filtration is even faster in the naturally perfused limb because rise in flow rate subsequent to arteriolar dilation augments the rise in capillary hydrostatic pressure produced by venous constriction. Acetate, then, is another agent which demonstrates the independence of arteries and veins (9) and the importance of the ratio of arterial to venous resistance in fluid transport across capillary membranes (4).

Except for $NaHCO_3$, the effects of the salts on total coronary resistance are similar to their effects on total forelimb resistance. Na acetate, Na citrate, and $NaNO_2$ decrease total coronary resistance, whereas Na lactate and NaCl are without effect. The changes are manifest at infusion rates below those which affect rate, QT interval, or per cent of a minute spent in electrical systole. This suggests that the agents may affect vessels without [illegible] in myocardial metabolism or intramyocardial pressure. [illegible] the response to Na bicarbonate differs from [illegible] the forelimb is not apparent.

[illegible] study provides little information concerning the [illegible] mechanism of anion arteriolar dilation. The [illegible] measurably effect plasma hydrogen or potassium concentration. The fact that excess calcium ion [illegible] both preparations (1, 2) suggests the possibility [illegible] the citrate ion dilates through its ability to [illegible] calcium.

[illegible] that local elevation of hydrogen ion concentration [illegible] produces active arteriolar dilation. All [illegible] decrease small vessel resistance. The resistance

TABLE 6. *Effects of intracoronary infusion of isosmotic solutions of sodium salts on heart rate and QT interval*

Solution	Control		2.6 ml/min	
	Heart rate, beats/min	QT interval, sec	Heart rate, beats/min	QT interval, sec
NaCl	124 (12)	0.32 (4)	122 (12)	0.32 (4)
$NaHCO_3$	113 (8)	0.32 (8)	115 (8)	0.32 (8)
$NaNO_2$	116 (7)	0.31 (7)	113 (7)	0.32 (7)
Na lactate	114 (8)	0.31 (8)	112 (8)	0.31 (8)
Na acetate	124 (8)	0.29 (8)	116 (8)	0.30 (8)
Na citrate	138 (8)	0.26 (8)	126 (8)	0.30 (8)

Figures in parentheses refer to numbers of experiments from which averages were derived.

decrease may be attributed entirely to the hydrogen ion in the cases of HCl, HNO_3, and lactic acid because chloride, nitrate, and lactate are by themselves ineffective. The difference between the responses to $NaNO_3$ and HNO_3 is particularly revealing since milliequivalent infusion rates of the two agents are the same. In the cases of pyruvic, acetic, and citric acids, both the hydrogen ion and the anion probably contribute to the response. However, quantitative comparisons of the responses to these acids and their corresponding salts are complicated by differences in milliequivalent infusion rates, by elevation of aortic pressure in the cases of pyruvic and citric acids, and by limb tetany in the case of citric acid. Hence, the reason why acetic acid does not dilate more than Na acetate is not clear.

Rise of arterial resistance during infusion of acid probably results, at least in part, from passive constriction of 0.5–5.0-mm-diameter arteries subsequent to fall of arterial transmural pressure. Rise of venous resistance, when it occurs, may result from increased levels of catecholamines subsequent to entrance of the acid into the general circulation. Acidosis is known to elevate catecholamine blood levels.

All the acids except nitric acid appear to liberate potassium from red cells because plasma potassium concentration rises in the perfusion system before the blood reaches the limb. This may in part explain the dilatory effect of the hydrogen ion because infusion of small quantities of potassium reduces small vessel resistance in this preparation (1). That some other mechanism is also involved in the dilation seems clear, because the magnitude of the resistance response is greater than is to be expected from the rise of potassium concentration, and because nitric acid dilates in the absence of a rise in the concentration of potassium.

The effects of acids on blood vessels have been studied by a number of investigators (10). Green and collaborators (10, 11) found that intracoronary injection of HCl caused vasodilation in both skin and muscle. The studies were complicated, however, by inclusion of a citrate-containing buffer in the injection, by systemic hypotensive toxicity, and nonsteady state conditions. Johnson (12) found that infusion of lactic acid into the

superior mesenteric artery decreased the resistance to blood flow through the intestinal vascular bed. The infusate was, however, hypertonic to plasma.

For a given pH reduction, the acids studied produced a much greater decrease of resistance than inhalation of CO_2. We have previously reported that inhalation of 20% CO_2 has very little effect on resistance to flow through the forelimb (13) or kidney (14), either before or after suppression of the sympathicoadrenal system. Further, perfusion of the forelimb with bloods containing equivalent O_2 contents but differing CO_2 contents fails to produce regular changes in resistance to flow (unpublished data). Crawford et al. (15) also question the importance of the effect of CO_2 excess on blood vessels.

REFERENCES

1. Overbeck, H. W., J. I. Molnar, and F. J. Haddy. *Am. J. Cardiol.* 8: 533, 1961.
2. Scott, J. B., E. D. Frohlich, R. A. Hardin, and F. J. Haddy. *Am. J. Physiol.* 201: 1095, 1961.
3. Frohlich, E. D., J. B. Scott, and F. J. Haddy. U. S. Army Medical Research Laboratory report no. 516, Nov. 3, 1961, Fort Knox, Ken.
4. Haddy, F. J., J. I. Molnar, and R. W. Campbell. *Am. J. Physiol.* 201: 631, 1961.
5. Hardin, R. A., J. B. Scott, and F. J. Haddy. *Am. J. Physiol.* 199: 163, 1960.
6. Scott, J. B., R. A. Hardin, and F. J. Haddy. *Am. J. Physiol.* 199: 765, 1960.
7. Haddy, F. J., P. Gordon, and D. A. Emanuel. *Circulation Research* 7: 123, 1959.
8. Bellet, S., S. V. Guzman, J. W. West, and D. M. Avtado. *Am. J. Med. Sci.* 233: 286, 1957.
9. Haddy, F. J. *Am. J. Physiol.* 198: 161, 1960.
10. Kester, N. C., A. W. Richardson, and H. D. Green. *J. Physiol.* 169: 678, 1952.
11. Deal, C. P., Jr., and H. D. Green. *Circulation Research* 2: 138, 1954.
12. Johnson, P. C. *Am. J. Physiol.* 199: 311, 1960.
13. Fleishman, M., J. Scott, and F. J. Haddy. *Circulation Research* 6: 602, 1957.
14. Emanuel, D. A., M. Fleishman, and F. J. Haddy. *Circulation Research* 6: 607, 1957.
15. Crawford, D. G., H. M. Fairchild, and A. C. Guyton. *Am. J. Physiol.* 197: 613, 1959.

[GANN, Vol. 43; Dec., 1952]

THE PRODUCTION OF GASTRIC LESIONS IN THE RAT BY ACETIC ACID FEEDING.*

(WITH PLATE XIV)

KAZUO MORI

(From the Laboratory of Medical Zoology, Showa Medical School, Tokyo)

The development of gastric lesions in the rats receiving diets containing acetic acid was discovered in this laboratory. And the results are reported in the following lines.

EXPERIMENTAL RESULTS

Experimental 1: 20 albino rats were maintained on rice, to which glacial acetic acid was mixed evenly. The amount of acetic acid added was 50 cc per 1 kg of rice through out the experiment. Usually, a small amount of cod liver oil was introduced along with acetic acid on the assumption that the dissipation of acetic acid from the surface of rice grains may be somewhat hindered by so doing. No attempt was made to estimate the actual amount of acetic acid ingested by the rats as the volatility of acetic 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.

Under the above experimental conditions, 10 rats were killed by carotid bleeding on the 30th day, and the stomachs were removed and placed in a fixative. After slight fixation, the stomachs were cut along the greater curvature. The tissues were embeded in paraffin and sectioned and stained routinely with hematoxylin and eosin.

In 3 of the 10 cases, a few umbilicate lesions were evident, and in 3 others slight thickening of the wall was seen in the forestomach, and the remaining 4 cases showed no gastric change. Other 5 rats were sacrificed on the 166th day, followed by autopsy. In 4 of the 5 cases, the umbilicate lesions or papillomatous growths were demonstrated in the forestomach.

All other rats were killed on the 200th day and the majority of them showed more or less definite gastric lesions as may be seen from the following table (Table 1):—

* Aided by a Scientific Research Encouragement Grant from the Ministry of Education.

Table 1

Rat No.	Sex	Period of Feeding (Days)	Body Weight (g)			Liver Weight (g)	Spleen Weight (g)	Gastric Lesions
			Initial	Maximum	Final			
1	♂	30	70	95	95	4.8	0.5	±
2	♂	30	65	85	80	4.2	0.5	−
3	♂	30	68	82	82	4.4	0.8	−
4	♂	30	60	65	65	3.8	0.4	−
5	♂	30	72	90	90	4.5	0.6	±
6	♂	30	65	80	80	3.5	0.4	±
7	♂	30	75	105	100	5.2	0.6	+
8	♂	30	68	85	82	4.0	0.4	+
9	♂	30	65	88	88	3.7	0.6	+
10	♂	30	65	85	85	5.0	0.6	−
11	♀	166	117	175	150	7.8	0.5	+
12	♂	166	87	145	120	8.0	0.4	−
13	♂	166	152	202	162	8.2	1.0	+
14	♀	166	95	167	152	8.3	0.6	+
15	♀	166	97	159	110	4.0	0.6	+
16	♀	200	130	205	200	8.8	0.8	+
17	♀	200	117	195	170	8.2	0.6	+
18	♀	200	122	203	203	8.8	0.8	+
19	♀	200	152	197	168	8.2	1.0	+
20	♀	200	95	170	170	7.6	0.6	+

In expressing the nature of gastric lesions the use was made of plus (+), plus-minus (±) and minus (−) signs indicating, respectively, umbilicate lesions, acanthosis or hyperkeratosis and macroscopically normal. In all cases, these diagnoses were comfirmed by microscopical examination.

Three examples of the gastric lesions are illustrated in Fig. 1.

In the above experiments, it is demonstrated that the gastric changes in the rats will be produced within 30 days after the beginning of the experiment. No remarkable change was found in other organs.

Experiment 2: In another experiment, in which the amount of acetic acid was greatly reduced in the beginning of the experiment, and the gastric changes were less remarked than in the above experiment. In this group, the amount of acetic 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 50 cc per 1 kg. Duration was 10 cc per 1 kg of rice for 18 days, 20 cc for 157 days and 50 cc for the rest of the experimental period. On the 200th day after the commencement of the experiment one rat died with umbilicate lesions in the forestomach. On the 250th day, half of the remaining rats were killed and the rest of animals were sacrificied on the 325th day, performing autopsy respectively. The results of the above experiment are

shown in the following table (Table 2).

Table 2.

Rat No.	Sex	Period of Feeding	Body Weight (g)			Liver Weight (g)	Spleen Weight (g)	Gastric Lesions
			Initial	Maximum	Final			
21	♂	200	102	102	65	6.2	0.4	+
22	♂	250	100	155	130	7.2	0.6	±
23	♂	250	142	167	130	7.1	0.5	+
24	♂	325	130	190	190	9.0	0.9	±
25	♂	325	140	170	137	7.6	0.8	−

In this table, 2 of the 5 cases showed umbilicate lesions scattered in the forestomachs and 2 others demonstrated slight hyperkeratosis and the remaing 1 case was macroscopically normal. In the above results, it is demonstrated that time required for the production of gastric changes is greatly prolonged when the amount of acetic acid was reduced.

Pathological Note

Grossly, in the forestomachs, the walls were opague, gray or white, indurated and rigid. The serosa, however, was glistening and smooth except at the base of gastric lesions where the wall was often retracted. The limiting ridge was prominent and often beaded. The gastric lesions varied from small, pin-point to large excrescences, and usually a small bloody red spot was detected at the top of the localized lesions.

Microscopically, the lesions were composed of proliferations of hyperplastic epithelium thrown into convoluted folds, and these proliferations were covered with keratin. A few rats in both experiments showed areas of gastritis and occasionally extensions of portions of the glandular mucosa into the submucosa.

Occasionally, spurs of hyperplastic epithelium extended downward from the papillomatous growths and penetrated the muscularis mucosa. Some of these were found deeply placed in the submucosa. In these areas mitotic figures were numerous, indicating a rapid growth of this lesion. Very often umbilicate lesions presented a defect in the keratin above it. This defect was regularly associate with an inflamatory focus in the epithelium and the underlying connective tissue. The basal layer was thickened.

The lamina propria and submucosa regularly showed an inflammatory reaction. The inflammatory cells consisted of neurotrophilic and eosinophilic leukocytes, lymphocytes, plasma cells and mononuclear leukocytes.

Discussion and Summary

Numerous investigators attempted to produce gastric lesions in the rat or mouse

with various diets or chemicals, as presented in a comprehe review by Sugiura.[1]

The criteria adopted in this paper for the gastric lesions of the forestomach were those of the rats which received tributyrin diet. In this laboratory, more than one hundred rats were fed for 500 days with the rice diet containing 5-10 per cent of butyric acid with a small amount of cod liver oil, supplemented with green vegetables just same as the above experiment. The results were almost similar to those obtained by Salmon and Copeland.[2,3] In the experiment, the amount of the butyric acid was reduced to the level of 5-10 per cant, less than half of the amount cited by Salmon, and few rats died during the early course of the experiment. And the stomachs of all rats fed for more than 50 days showed ulcers or small discrete papillomas. As indicated by Salmon, in the rats of this laboratory numerous cysts were found penetrating the muscularis mucosa, and these cysts were lined with stratified squamous epithelium and contained keratin in a lamellated fibrillary arrangement. These results of butyric acid feeding will be reported elsewhere.

In the case of the rats fed acetic acid, no cyst or nodule containing keratin was demonstrated in the forestomach. But, except the absence of keratin cyst in the forestomach, the other gastric lesions caused by acetic acid were similar but less marked than those due to butyric acid.

It is noteworthy that the two chemicals in the low fatty acid group similarly produce the gastric lesions of rats by oral administration. A question remaines as to how propionic acid, one of the low fatty acids, act on the forestomach of the rats, and the inverstigation is now under way in this laboratory.

In conclusion the author wishes to express his sincere thanks to Dr. Sanji Kishi, Head of Biochemical Section of this School, for his valuable suggestions.

Literature Cited

1) Sugiura, K., Cancer Research, Vol. 2,770 (1942)
2) Salmon, W. D., and Copeland, D. H., Jour. Nat. Cancer Inst., Vol. 10, 361 (1949).
3) Salmon, W. D., and Coodman, J. G., J. Nutrition, Vol. 13, 477 (1937). cited in (2).

[GANN, Vol. 41; December, 1953]

INHIBITION OF EXPERIMENTAL PRODUCTION OF LIVER CANCER BY ADDITION OF ACETIC ACID TO THE DIET*

KAZUO MORI

(From the Laboratory of Medical Zoology, Showa Medical School, Tokyo)

Since the discovery of the production of the liver cancer using certain azo-compounds by Sasaki and Yoshida,[1] and Kinoshita,[2] numerous attempts to inhibit the liver cancer resulting from the feeding of these dyes have been demonstrated by many investigators. And their results have indicated the possible existence of liver cancer inhibiting substances in vegetable and animal materials.[3-11] Several of these expriments show that the diet may have some effect on the experimental liver cancer production, but, so far as the author is aware, no such drastic inhibition as effected by feeding liver[3,5,11,13] and kidney[8] has ever been brought about by any other dietary means. The inhibiting effect by feeding liver was to such an extent as to prevent even the development of cirrhosis and to keep liver macroscopically normal for 150 days and more. The present experiment has demonstrated that the experimental production of liver cancer is inhibited by the diet supplemented with acetic acid. The details of this experiment are given in the following pages.

EXPERIMENTS

Three groups of male and female albino rats ranging from 60 to 80 g in weight were used for this experiment.

The first group: 60 rats were maintained on unpolished rice (1 kg) evenly mixed with p-dimethylaminoazobenzene (butter yellow) (0.6 g) dissolved in glacial acetic acid (50 cc).

The second group: 10 rats were kept on the acetic acid butter yellow diet, but in this group acetic acid was added at the level of 2.5 per cent. In these groups (groups 1 and 2), when the p-dimethylaminoazobenzene was dissolved in glacial acetic acid, the color of the solution turned into crimson red. But this red color again turned back to yellow within five minutes when the butter yellow-acetic acid solution was mixed with rice.

The third group (control): 40 rats were fed on the mixture of unpolished rice (1 kg) and methanol (50 cc) in which p-dimethylaminoazobenzene (0.6 g) was dissolved.

In view of the deficient nature of the diet, a small amount of the cod liver oil was added to each. And all experimental animals were given green vegetables once a week and water ad libitum. Some of the rats died early in the course of the experiment, too early to show any relevant change, and they were discarded. Among the first group, 10 on the 120th day, 15 on the 140th day and 25 rats on the 165th day were killed by carotid bleeding and autopsied. The second and third groups were terminated on the 150th day after the commencement of experiments by sacrificing all the rats in order to make a final comparison of liver changes between these groups.

The results of the above experiments are summarized in Table 1, where the nature of liver findings is tabulated in the following four degrees: liver cancer, cirrhotic change only, slightly uneven surface without extensive proliferation of connective tissue, and macroscopically normal. The identification of these liver findings has been described in detail in the publication cited.[5]

In the first group, 9 (90.0%) among the total 10 rats which were sacrificed on the 100th experimental day showed apparently normal liver and in the remaining 1 case (10.0%) the liver change was not advanced beyond the stage of somewhat granular and uneven surface. 8 (53.5%) among the total 15 rats which were killed on the 140th day showed macroscopically normal livers, 4 rats (26.6%) showed slightly granular livers, and in the remaining 3 cases, the localized or annular cirrhosis of the liver was seen. Among the total 25 rats which were killed on the 165th day, 21 (84.0%) showed macroscopically normal livers, and the liver surface in the other 3 cases (12.0%) was slightly granular, and only in the remaining 1 case (4.0%) cirrhotic change was advanced. No liver cancer was produced in the rat of this group.

A somewhat notable feature of the experimental results was that the slight splenic enlargement was indicated in the rats fed on acetic acid diet at the level of 5 per cent, notwithstanding the absence of notable change in the liver size as shown in Table 2. The average of the ratio of the spleen to the total body weight of the experimental animals was 1.07, more than twice that of the normal (0.3–0.43).

Against the results of the first group, no inhibition of the production of liver cancer was demonstrated in the second group in which the dye diet was mixed with the acetic acid at the level of 2.5 per cent. As is shown in Table 1, the second group included 4 cases (40.0%) of liver cancer accompaning cirrhosis, 4 cases (40.0%) of typical cirrhosis and the remaining 2 cases were beyond the stage of somewhat granular and uneven surface.

Then, the findings in the third group (control) were as follows: 9 cases (29.0%) of macroscopically normal, 5 cases (16.3%) of uneven surface, 7 cases (22.6%) of typical cirrhotic and 10 (32.3%) of liver cancer accompaning annular cirrhosis.

The average amount of food intake per rat per day in the first group (acetic acid group) was 7.7 g and that of the third (control) was 8.9 g. And it is thought that the difference within this range in the amount of food intake will not affect

* Aided by a Scientific Research Encouragement grant from the Department of Education.

Comparison of Liver Changes between Acetic Acid Fed and Control Dye-diet Groups.

Experimental Group	Experimental Days	No. of rats	Liver Findings: Macroscopically Normal	Liver with Slightly uneven surface	Cirrhotic Liver	Liver Cancer
First Group Dye-diet with 5% acetic acid	100	10	9 (90.0%)	1 (10.0%)	0	0
	140	15	8 (53.5%)	4 (26.6%)	3 (20.0%)	0
	165	25*	21 (84.0%)	3 (12.0%)	1 (4.0%)	0
Total		50	38 (76.0%)	8 (16.0%)	4 (8.0%)	0
Second Group Dye-diet with 2.5% acetic acid	150	10	0	2 (20.0%)	4 (40.0%)	4 (40.0%)
Third Group Dye-diet only (Control)	150	31	9 (29.0%)	5 (16.3%)	7 (22.6%)	10 (32.3%)

* The weights of the body, liver and spleen of these rats are show in Table 2.

the degree of the liver cancer production, according our experience of these ten years.

The results of these experiments described above clearly demonstrated that the acetic acid at the level of 5 per cent in the dye diet has inhibited markedly the production of liver cancer, but when the amount of acetic acid was reduced to 2.5 per cent in the diet, the inhibiting effect failed completely.

The above result leaves little doubt as to the marked inhibiting effect which acetic acid feeding at the level of 5 per cent exerts on the production of liver cancer by oral administration of p-dimethylaminoazobenzene. In this experiment, a question arose as to the possibility of butter yellow being destroyed while it is kept in contact with acetic acid in the diet mixture.

As described above, the glacial acetic acid, in which butter yellow was dissolved, mixed with rice and the rats were allowed to feed upon the mixture ad libitum. It is not entirely without reason to suspect that this carcinogen might suffer some chemical modification by the contact with glacial acid outside of the animal body, with the resultant loss of the carcinogenecity before it enters into the body of the animal. Here is the experimental datum, demonstrating that butter yellow is by no means destroyed by being kept mixed with acetic acid and rice. Out of 100 g of the acetic acid-butter yellow rice, 65 mg of butter yellow was indicated (theoretically, a 100 g portion of the original mixutre contains 60 mg of butter yellow), and the crystals isolated had M. P. 115°C, agreeing with that of pure p-dimethylaminoazobenzene. The method of recovery of the azo dye used here was the same

Table 2. Body, Liver and Spleen Weights and their Ratios in the rats fed Acetic Acid Dye Diet killed on the 165th day.

Rat No.	(A) Body weight (g)	(B) Liver weight (g)	(C) Spleen weight (g)	Liver ratio B/A x100	Spleen ratio C/A x100	Liver Finding
1	95	5.5	1.0	5.8	1.5	Macroscopically normal
2	110	6.8	1.7	6.2	1.5	〃
3	130	8.3	1.5	6.4	1.1	〃
4	140	6.9	1.6	4.6	1.1	〃
5	145	8.5	2.2	5.8	1.5	〃
6	155	7.8	1.4	5.0	0.9	〃
7	160	7.1	1.7	4.4	0.1	〃
8	165	7.9	2.1	4.8	1.3	〃
9	170	9.0	1.9	5.3	1.1	〃
10	170	9.2	2.3	5.4	1.3	〃
11	175	9.0	1.7	5.1	1.0	〃
12	180	7.7	2.0	4.3	1.1	〃
13	180	10.0	1.5	5.6	0.8	〃
14	185	9.5	0.8	5.1	0.5	〃
15	190	8.5	1.0	4.5	0.5	〃
16	190	10.5	1.7	5.5	0.9	〃
17	200	11.4	2.5	5.7	1.2	〃
18	210	10.1	1.5	4.8	0.7	〃
19	215	10.8	1.8	5.0	0.8	〃
20	220	14.0	2.2	6.3	1.0	〃
21	235	10.6	2.0	4.5	0.8	〃
22	95	7.8	0.9	8.2	0.9	uneven surface
23	130	9.4	1.9	7.2	1.4	〃
24	175	10.2	1.5	5.8	0.9	〃
25	120	9.7	1.8	8.0	1.5	Liver cirrhotic

as to that of Nakahara and Kishi.[15]

Nakahara and Fukuoka,[16] and Miyaji and Nakajima[17] have demonstrated that the marked increase in the catalase activity of the liver of rats fed certain diet, for instance the diet containing the beef liver powder, will reduce the production of liver cancer. The average of catalase activity of ten rats fed the acetic acid diet (5 per cent) for the period of 30 days was 6.8 cc in oxygen unit, while it was 5.8 cc in rats of normal diet.[18]

Recently, the development of a severe hyperplasia of the forestomach in rats receiving diets containing acetic acid only was observed in this laboratory.[19] But these gastric lesions were less remarked or not produced in the rats which were fed acetic acid-butter yellow diet (group 1) than the rats fed acetic acid only.

Discussion and Conclusions

Experimental studies on the dietary influence on the genesis of cancer have so far yielded a few significant leads,[13] and yet they remain a problem one of the most important in the field of cancer research. In the previous papers,[3,5,8] it was demonstrated that the liver and kidney feeding brought about a definite inhibition of liver cancer production by p-dimethylaminoazobenzene. In the present investigation dealing with liver cancer production by p-dimethylaninoazobenzene, it has been demonstrated that the acetic acid feeding inhibited the process of liver cancer production, at the level of 5 per cent concentration in the diet. And it is thought that this result was not due entirely to lowered intake of the carcinogen. However no inhibition of liver cancer production took place with the concentration of the acetic acid was reduced to 2.5 per cent.

It is of special interest to note that the acetic acid is the simplest chemical among the dietary supplements hitherto used, such as, corn oil[20,21,22], olive oil[22], lauric acid[23,24], choline[25], riboflavin[26,27,28], thiosalicylic acid, thiouracil[27] and others[29], by which the marked inhibition or retardation of the experimental liver cancer production was brought about.

How the acetic acid feeding brings about the inhibition of cirrhosis or hepatoma production is indeed a complex problem, and the investigation is now under way in this laboratory.

Literature

1) Sasaki, T., and Yoshida, T., : Virchow's Archiv, Bd. 251, 175 (1935).
2) Kinoshita, R., Trans. Japan. Pathol. Soc., Vol. 27, 665 (1937).
3) Nakahara, W., Mori, K. and Fujiwara, T., Gann, Vol. 32, 465 (1938).
4) Kinoshita, R., Gann, Vol. 33, 225 (1939).
5) Nakahara, W., Mori, K., and Fujiwara, T., Gann. Vol. 33, 406 (1939).
6) Ando, T., Gann, Vol. 32, 252 (1939), Vol. 33, 229 (1939), Vol. 34, 356 (1940), Vol. 35, 41, 58, and 562 (1942).
7) Sato, H., and Morigami, S., Gann, Vol. 35, 301 (1941).
8) Mori, K., Gann, Vol. 35, 86 and 106 (1941).
9) Nakahara, W., Fujiwara, T., and Mori, K., Gann, Vol. 33, 57 (1939).
10) Vassiliadis, H. C., Amer. J. Cancer, Vol. 39, 377 (1940).
11) Mori, K., and Nakahara, W., Gann, 37, 453 (1943).
12) Morigami, S., et al., Gann, Vol. 35, 65 (1941).
13) Nakahara, W., Kishi, S., and Mori, K., Gann, Vol. 37, 472 (1943).
14) Ugami, S., Proc. Imp. Acad., Vol. 19, 95 (1943).
15) Kishi, S., and Nakahara, W., Gann, Vol. 36, 361 (1942).
16) Nakahara, W., and Fukuoka, F., Gann, Vol, 38, 340 (1941) and Vol. 39, 40 (1948).
17) Miyaji, T., and Nakajima, T., Gann, Vol. 39, 35 (1948).
18) Mori, K., Gann, Vol. 43, 431 (1952).
19) Mori, K., Gann, Vol. 43, 443 (1952).

20) Kline, B. E., Miller, J. A., and Rusch, H. P., Cancer Res., Vol 5, 641 (1945).
21) Miller, J. A., Kline, B. E., and Rusch, H. P., Cancer Res., Vol. 6, 674 (1946).
22) Harris, P. N., and Clowes, G.H.A., Cancer Res., Vol. 12, 471 (1952).
23) Kline, B. E., Miller, J. A., Rusch, H.P., and Baumann, C. A., Cancer Res., Vol. 6, 1 (1946).
24) Ibidem, Cancer Res., Vol. 6,5 (1946).
25) Dyer, H. M., Jour. Nat. Cancer Inst., Vol. 11, 1073 (1951).
26) Giese, J. E., Clayton, C. C., Miller, E. C. and Baumann, C. A., Cancer Res., Vol. 6, 679 (1946).
27) Griffin, A. C., Clayton, C. C., and Baumann, C. A., Cancer Res., Vol. 9, 82 (1949).
28) Harris, P. N., Krahl, M. E., and Clowes, G.H.A., Cancer Res., Vol. 7, 162 (1947).
29) Greenstein, J. P., and Haddow, A., Advances in Cancer Research, Vol. 1, Academic Press, N. Y. (1953).

In Vitro Incorporation of ^{14}C Labeled Precursors into Swine Coronary Arterial Phospholipids

Experimental atherosclerosis in the rabbit is associated with an increased rate of synthesis of phospholipids, particularly phosphatidyl choline, in the aortic wall[1-4]. Different types of phospholipids have been shown to vary in their capacity to stabilize cholesterol emulsions[5]. It is possible that differences in content or synthesis of the various types of phospholipids in the arterial wall could be a factor in the differing susceptibilities of some types of arteries or some species to atherosclerosis. This possibility was explored in the present investigation by studying the coronary arteries of swine in comparison to the pulmonary and other arteries and human peripheral arteries of previous studies[1-6].

Materials and methods. Hearts and portions of the aorta and pulmonary arteries were obtained within 30 min of slaughter from 6-month-old, male, grain fed Hampshire swine. The intima-media portions of the coronary and other arteries were placed in an isotonic Krebs-Ringer-glucose solution at pH 7.4, and after addition of 20 µc of either formate-^{14}C, acetate-1-^{14}C, glycerol-1-3-^{14}C, choline-1-2-^{14}C, serine-1-^{14}C or ethanolamine-1,2-^{14}C (Nuclear-Chicago, adjusted to specific activity of 1.03 mc/mM) to each, the flasks were incubated for 4 h in an air atmosphere at 37.5 C in a Dubnoff metabolic shaking incubator. A total of 4 incubations for each artery were done with each ^{14}C labeled precursor. Carbon dioxide was collected in a center well containing 20% NaOH, and the $^{14}CO_2$ radioactivity determined as previously described[1].

Washing the tissues free of radioactive precursor, extraction of the lipids and separation of the phospholipids by thin layer chromatography were all done as previously described[1]. After elution of the phospholipid fractions from the silica gel chromatograms were analyzed for phosphorous content[7]. Other aliquots were assayed for radioactivity using a mixture of 2,5-Diphenyloxazole and 1,4-*bis*-2-(5-Phenyloxazolyl)-Benzene in toluene scintillator and a Packard Model 3314 Liquid Scintillation spectrometer[4].

Results and discussion. The amounts of the individual major phospholipids in the coronary arteries in mg/g dry weight of arteries ± S.D. were as follows: sphingomyelin 0.7 ± 0.2, phosphatidyl choline 2.8 ± 0.6, phosphatidyl serine 0.8 ± 0.3 and phosphatidyl ethanolamine 1.3 ± 0.4. The amounts of these phospholipids were not significantly different in the coronary or pulmonary arteries or in the aorta; incubation with the different labeled precursors also had no effect upon the amounts of any of these phospholipid classes.

The $^{14}CO_2$ evolved in dpm/mg dry weight of artery for the coronary and pulmonary arteries after incubation with each of the ^{14}C labeled precursors is indicated in Table I. Oxidation of carbon 1 of serine to $C^{14}O_2$ was greater than that for the labeled carbon atoms of the other amino-alcohol substrates utilized. The amounts of $^{14}CO_2$ evolved from the aorta were significantly lower than those from the pulmonary and coronary arteries.

The specific activities of the major coronary arterial phospholipids after incubation with the different ^{14}C labeled precursors are indicated in Table II. The nitrogenous bases appeared to be incorporated to a much greater degree than formate, acetate or glycerol. This was probably due to the more selective utilization of the bases for phospholipid synthesis, and also to the greater oxidation of formate and acetate to CO_2. Sphingomyelin showed a relatively low degree of incorporation with all the precursors, including the choline-^{14}C. Phosphatidyl serine was most highly labeled when incubated with the serine-^{14}C or formate-^{14}C, incorporation of the latter occurring probably by fixation of the one carbon fragment to glycine to form serine.

Phosphatidyl choline was significantly labeled after incubation with choline-^{14}C and with ethanolamine-^{14}C

Table I. $^{14}CO_2$ evolved during incubation of arterial tissue slices with ^{14}C-labeled substrates[a]

Substrate	Coronary	Pulmonary	Aorta
Formate	1,150 ± 204	[illegible] ± 81	[illegible] ± 22
Acetate	2,500 ± 224	2,900 ± 800	[illegible] ± 150
Glycerol	100 ± 30	[illegible] ± 25	8 ± 10
Choline	25 ± [illegible]	2 ± [illegible]	[illegible]
Serine	177 ± 28	250 ± [illegible]	81 ± 15
Ethanolamine	22 ± 5	[illegible] ± [illegible]	[illegible] ± 2

[a] dpm $^{14}CO_2$/mg dry weight of artery.

[1] A. [illegible] and A. L. Day and D. B. Zilversmit, Circulation Res. [illegible], 32 (1963).
[2] A. L. Day and [illegible] K. W. Kinsell, Circulation Res. [illegible], 878 (1963).
[3] M. Nakayama, [illegible] Sasaka and M. Nakamura, Atherosclerosis [illegible], 17 (1963).
[4] [illegible]
[5] [illegible]
[6] [illegible]
[7] [illegible]

Table II. Specific activities of major coronary arterial phospholipids after incubation with ^{14}C labeled substrates[a]

Substrate	Sphingomyelin	Phosphatidyl choline	Phosphatidyl serine	Phosphatidyl ethanolamine
Formate	[illegible]	[illegible]	[illegible]	[illegible]
Acetate	[illegible]	[illegible]	[illegible]	[illegible]
Glycerol	[illegible]	3,516 ± 312	[illegible]	[illegible]
Choline	[illegible]	[illegible]	[illegible]	[illegible]
Serine	[illegible]	[illegible]	[illegible]	[illegible]
Ethanolamine	[illegible]	[illegible]	[illegible]	[illegible]

[a] DPM/mg phosphorous ± S.D.

[illegible]ocating that both the de-novo and the transmethyla-[illegible] pathways are active in the swine arteries.

[illegible]he patterns of incorporation in the aorta and pulmonary arteries were not significantly different from [illegible] of the coronary arteries; the degrees and distribution of radioactivity incorporation were similar to those [illegible]d on incubation of the same precursors with rabbit [illegible]s or human platelet [illegible]. There is therefore evidence from these experiments to suggest that [illegible] [illegible]tions susceptibilities to [illegible] in the coronary [illegible] and aorta vs. the pulmonary artery are attributable to differences in phospholipid metabolism of these [illegible].

Summary. Der Grad d[illegible] Einbaus von ^{14}C-markierten Präkursoren in die Arterienphospholipide des Schweins war folgender: Cholin > Aethanolin > Serin > Glycerin > Formiat > Azetat. Bei Bebrütung von Arterien mit ^{14}C-Cholin oder ^{14}C-Aethanolamin trat ein deutlich erhöhter Einbau von ^{14}C in das Phosphatidylcholin auf.

R. J. Morris

Department of Pathology,
Los Angeles County/University of Southern California General Hospital,
Department of Pathology, UCLA, and the
UCLA School of Medicine,
Los Angeles, California, USA, 28 April 1979.

* This investigation was supported by grant No. 5K01-HE10172-03 from the National Heart and Lung Institute, U.S.P.H.S.

Life Sciences Vol. 8, Part II, pp. 613-616, 1969. Pergamon Press
Printed in Great Britain.

INCORPORATION OF FORMATE-^{14}C, ACETATE-1-^{14}C CHOLINE-1,2-^{14}C AND ETHANOLAMINE-1-2^{14}C INTO RABBIT OVARIAN PHOSPHOLIPIDS*

Robert J. Morin

Division of Reproductive Biology and
Departments of Pathology, Los Angeles County
Harbor General Hospital, Torrance, California
and the U.C.L.A. School of Medicine,
Los Angeles, California

(Received 10 February 1969; in final form 13 March 1969)

Although the content and metabolism of cholesterol and total fatty acids in the ovary has been the subject of several investigations (1-3), there have been only a few studies of ovarian phospholipids (4-5). The total phospholipids of the rabbit ovary during pregnancy appear to gradually increase up to the fourteenth to sixteenth days, after which the phospholipid concentration decreases (4). In the sow, Bloor (5) found that high levels of ovarian phospholipids are attained at ovulation and continue at this high level throughout pregnancy.

In a more recent study, ovarian phosphatidyl choline in particular was found to be increased in amount during pregnancy, and acetate-1-^{14}C incorporation into phosphatidyl choline was also increased (6). Of the major fatty acids of phosphatidyl choline, only palmitic acid showed a significantly increased radioacetate incorporation with pregnancy.

The objectives of the present experiments were to further investigate phospholipid metabolism in rabbit ovaries by observing the *in vitro* incorporation of several different precursors.

*This investigation was supported by a grant from the Ford Foundation

Materials and Methods

Sixteen female New Zealand white rabbits, four months old, and fed a stock commercial diet (Purina) since weaning, were divided into four groups of four each. The rabbits were anesthetized by I.V. injection of sodium pentobarbital, the ovaries excised from each animal, sliced at 0.4 mm. intervals with a McIllwain Tissue Chopper and then placed in a flask with 10 ml. of Tyrode's solution. After addition of 20 μc of either formate-^{14}C, acetate-1-^{14}C, choline-1,2-^{14}C or ethanolamine-1,2-^{14}C (Nuclear Chicago, all adjusted to specific activity of 1.03 mc/mM) to each, the flasks were incubated for two hours in an air atmosphere at 37.5°C in a Dubnoff metabolic shaking incubator.

Collection and assay of $^{14}CO_2$, washing the tissues free of radioactive precursor, extraction of the lipids, and separation of the phospholipids by thin layer chromatography were all done as previously described (6,7). Because of the small amounts and impurity of the phosphatidyl inositol fraction, this phospholipid was not included in the present study. After elution of the phospholipid fractions from the silica gel (7) aliquots were analyzed for phosphorous content (8). Other aliquots were assayed for radioactivity using a PPO-POPOP in toluene scintillator and a Packard Model 3314 liquid scintillation spectrometer (6).

Results and Discussion

The order of concentration of the individual ovarian phospholipids was phosphatidyl choline > phosphatidyl ethanolamine > phosphatidyl serine ≐ sphingomyelin > lysophosphatidyl choline. The amounts of each ovarian phospholipid have been given in a previous publication (6).

$^{14}CO_2$ production from formate-^{14}C was 2,340 ± 250 DPM/mg dry ovary; from acetate-^{14}C, 1420 ± 280; from choline-^{14}C, 96 ± 17; and from ethanolamine-1,2-^{14}C, 70 ± 12.

TABLE I

Specific Radioactivities of Individual Rabbit Ovarian Phospholipids after Incubation with the ^{14}C Labeled Precursors*

	Formate-^{14}C	Acetate-1-^{14}C	Choline-1,2-^{14}C	Ethanolamine-1,2-^{14}C
Lysophosphatidyl choline	723 ± 104	156 ± 29	6,150 ± 713	1,440 ± 167
Sphingomyelin	660 ± 95	621 ± 78	1,540 ± 125	887 ± 94
Phosphatidyl choline	756 ± 152	573 ± 80	15,050 ± 1,220	2,320 ± 255
Phosphatidyl serine	2,622 ± 380	714 ± 75	1,750 ± 183	722 ± 59
Phosphatidyl ethanolamine	870 ± 97	312 ± 42	1,010 ± 131	13,420 ± 1,270

*Specific activity is expressed as DPM/mg phospholipid ± standard deviation.

In Table I are given the specific radioactivities of the ovarian phospholipids after incubation with each radioactive substrate. Choline-^{14}C appeared to be most actively incorporated into the total ovarian phospholipids and formate and acetate showed the lowest incorporation. These differences may have been partially due to the lesser availability of substrate due to the more rapid oxidation of formate and acetate to CO_2 and also probably to the greater utilization of formate and acetate by other pathways, as compared to the more selective utilization of choline and ethanolamine in phospholipid synthesis.

Formate-^{14}C appeared to be incorporated primarily into phosphatidyl serine, most likely by fixation of the one carbon fragment to glycine, as has been demonstrated in other tissues (9,10).

Acetate-1-^{14}C was more uniformly incorporated into all the phospholipid classes, probably primarily into their fatty acid moieties.

Although choline-1,2-^{14}C was incorporated primarily into phosphatidyl choline, smaller amounts also appeared in the other choline-containing phospholipids (lysophosphatidyl choline and sphingomyelin) and also into phosphatidyl serine and phosphatidyl ethanolamine; this probably occurred via catabolism of choline to glycine, with subsequent synthesis of serine and ethanolamine.

Incorporation of ethanolamine-1,2^{14}C occurred primarily into phosphatidyl ethanolamine, with smaller but significant amounts incorporated into the choline-containing phospholipids, particularly phosphatidyl choline. This suggests that the enzyme system for the transmethylation of phosphatidyl ethanolamine to phosphatidyl choline is active in the rabbit ovary.

References

1. W. W. MARION and J. L. SELL, Poultry Sci., 42, 614 (1963).
2. K. J. RYAN and R. V. SHORT, Endoc., 78, 214 (1965).
3. R. T. HOLMAN and H. H. HOFSTETTER, J. Am. Oil Chem. Soc., 42, 540 (1965).
4. E. M. BOYD, J. Biol. Chem., 108, 607 (1935).
5. W. R. BLOOR, R. OKEY and G. W. CORNER, J. Biol. Chem., 86, 291 (1930).
6. P. J. MORIN, J. Reprod. Fert., 17, 111 (1968).
7. V. P. SKIPSKI, R. F. PETERSON and M. BARCLAY, Biochem. J., 90, 374 (1964).
8. D. B. ZILVERSMIT and K. A. DAVIS, J. Lab. Clin. Med., 35, 155 (1950).
9. W. SAKAMI, J. Biol. Chem., 176, 995 (1948).
10. P. SIEKEVITZ and D. M. GREENBERG, J. Biol. Chem., 186, 275 (1950).