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Gastric Absorption and Distribution of Acetylsalicylic Acid and Other Acidic Compounds in the Rat

CLARENCE H. MORRIS, JOHN E. CHRISTIAN, TOM S. MIYA, and WARREN G. HANSEN

Abstract □ A comparison was made of the gastric absorption and distribution of sodium acetylsalicylate-7-^{14}C with the absorption and distribution of sodium salts of other weakly acidic compounds. Each of the other compounds observed had an absorption pattern which was characteristic for the compound. Only sodium acetylsalicylate caused gastric lesions in the rat. The observations do not rule out the possibility that absorption characteristics of acetylsalicylic acid and its salts may be associated with their ability to cause gastric ulcers.

Keyphrases □ Acetylsalicylate-7-^{14}C, Na—absorption, distribution □ Acidic compounds—acetylsalicylate-7-^{14}C, Na—absorption, distribution comparison □ Gastric lesion production—acetylsalicylate-7-^{14}C, Na, acidic compounds □ TLC—analysis □ Autoradiography—analysis

It has been known for more than 50 years that oral administration of acetylsalicylic acid is followed by erosion of the gastric mucosa and bleeding from the site of the lesion (1). These aspirin-induced lesions have been observed in humans (1), dogs (2), cats (3), guinea pigs (4), and albino rats (5). A number of hypotheses have been suggested to explain the occurrence of these lesions. Davenport (6) and Martin (7) have suggested chemical models based upon the interaction of the compound with cellular constituents following its absorption

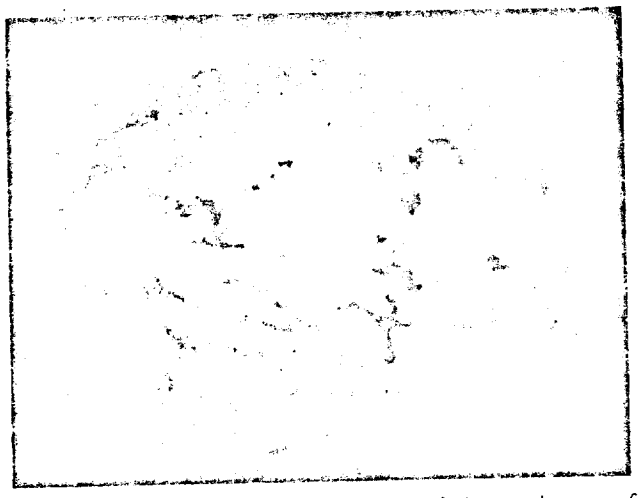

Figure 1—*Corpus mucosal surface showing lesions and areas of hemorrhage.*

into the cell. It became of interest to compare the absorption of aspirin, which causes ulceration and bleeding in the corpus portion of the rat stomach, with the absorption of other weakly acidic compounds which do not cause ulceration and bleeding.

MATERIALS AND METHODS

The radiochemical purity of sodium acetylsalicylate-7-^{14}C, sodium benzoate-7-^{14}C, sodium acetate-1-^{14}C, and sodium barbital-2-^{14}C (Tracer Labs, Waltham, Mass.) was established by TLC employing a stationary phase of silica gel G and a solvent system of low-boiling petroleum ether and 99% propionic acid. The location of the radioactive spots was determined by gross autoradiography.

All animals were fasted for 36 hr. prior to administration of the compound. Water was allowed *ad libitum* during the fast but was withdrawn after administration of the compound.

The compounds were administered orally to male Holtzman rats weighing between 150 and 275 g. (Holtzman Rat Co., Madison, Wis.) by means of a No. 16 curved steel oral catheter which had been dipped in mineral oil for lubrication. Each animal received a dose of 0.28 mmole of the selected compound per kg. of body weight. Each dose contained 30.5 μc. per kg. body weight of the ^{14}C-labeled compound diluted with the appropriate amount of carrier. The sodium acetylsalicylate was administered in 0.15 M citrate buffer, final pH 4.6. All other compounds were dissolved or suspended in a solution of 5% polyvinylpyrrolidone (PVP). Control animals received 0.5 ml. of the respective vehicle.

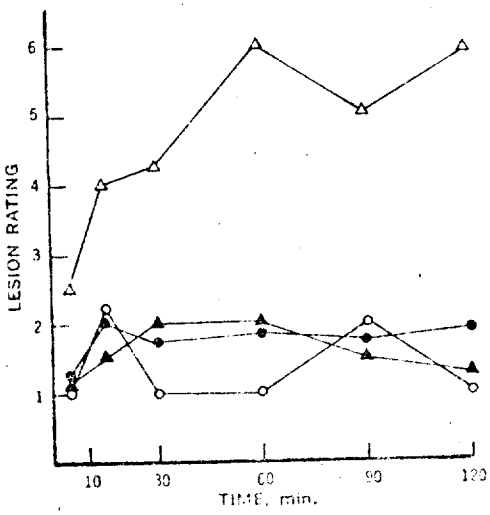

Figure 2—*Average gastric lesion rating of six animals per time interval following oral administration of: △, sodium acetylsalicylate; ○, sodium barbital; ●, sodium acetate; and ▲, sodium benzoate. All control animals had a rating of 1.*

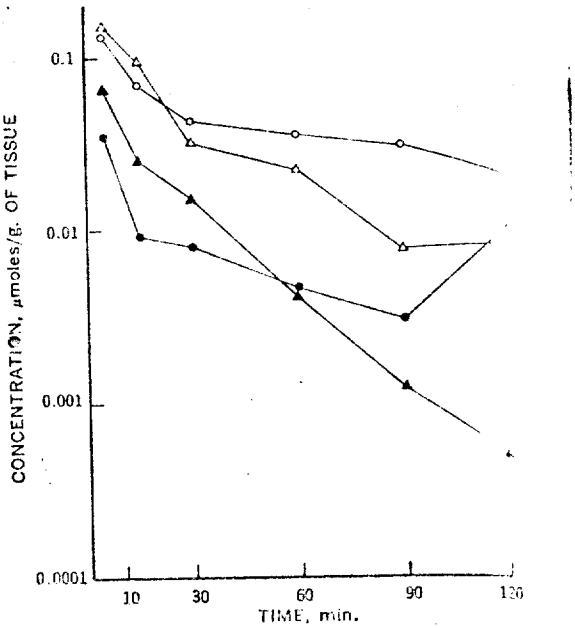

Figure 3—*Disappearance of labeled compounds from the corpus portion of the rat stomach. Key: △, sodium acetylsalicylate; ○, sodium barbital; ●, sodium acetate; and ▲, sodium benzoate. Each point represents the mean of three animals. To indicate the range of values around the averages shown in the plot, the minimum variation around any single point was 2.62 × 10^{-1}, 1.98 × 10^{-1}, and 2.24 × 10^{-1} (average 2.28 × 10^{-1}) μmoles per gram of tissue. The maximum variation around any single point was 1.94 × 10^{-3}, 1.05 × 10^{-3}, and 5.13 × 10^{-3} (average 4.95 × 10^{-3}) μmoles per gram of tissue.*

The rats were killed in groups of three by etherization at intervals of 5, 15, 30, 60, 90, and 120 min. following administration of each compound. The stomachs were removed, opened along the line of lesser curvature, stretched, and pinned on a large rubber stopper. The mucosal surface of each stomach was decontaminated with 0.9% NaCl, examined for the appearance of lesions, and rated on an 8-point scale developed by Morris *et al.* (5).

After being rated with respect to the severity of lesions, the stomachs were frozen in the stretched position with dry ice. Whole frozen stomachs were covered with plastic wrap (Saran), placed between two sheets of X-ray film (No Screen Medical, Eastman Kodak Co., Rochester, N. Y.), and stored in light, tight cassettes at −20° for 60 days for gross autoradiography.

Following exposure of the film, samples of frozen rumen (nonglandular portion of the rat stomach) and corpus (glandular portion of the rat stomach) were prepared for liquid scintillation counting (Tri-Carb liquid scintillation counter model 3003, Packard Instrument Co., La Grange, Ill.) as described by Morris *et al.* (5). The amount of compound present per gram of tissue was calculated for each sample according to the following expression:

$$\mu\text{moles of compound/g. of tissue} = \frac{\text{observed d.p.m.}}{(W)\,(S\text{-}I)\,(L)}$$

where: W = wet weight of the tissue in grams; $S.A$ = specific activity, d.p.m./mcg. of compound; and L = mcg. of compound/μmole.

RESULTS

Appearance of Lesions—Typical lesions as they appeared in the corpus portion of the rat stomach are shown in Fig. 1. It is of interest to note that the lesions appear in rows or furrows [illegible]

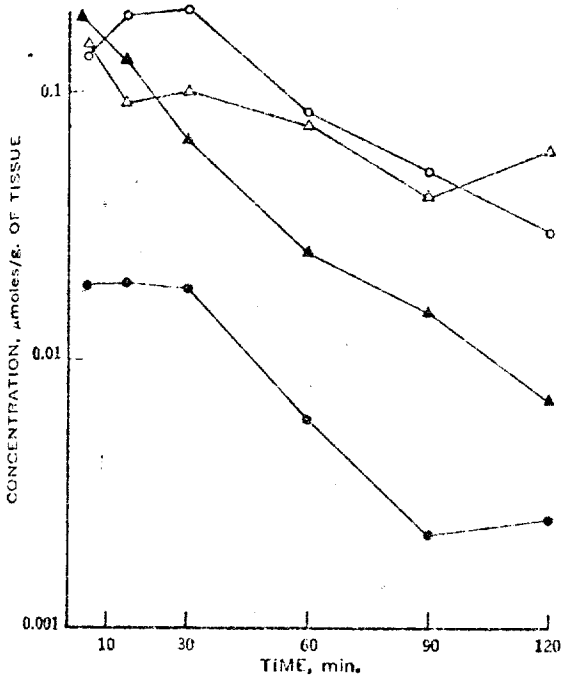

Figure 4—*Disappearance of labeled compounds from the rumen portion of the rat stomach. Key: △, sodium acetysalicylate; ○, sodium barbital; ●, sodium acetate; and ▲, sodium benzoate. Each point represents the mean of three animals. To indicate the range of values around the averages shown in the plot, the minimum variation around any single point was 4.27 × 10⁻², 3.94 × 10⁻², and 3.94 × 10⁻² (average 4.95 × 10⁻²) μmoles per gram of tissue. The maximum variation around any single point was 1.12 × 10⁻², 2.04 × 10⁻², and 4.6 × 10⁻² (average 1.2 × 10⁻²) μmoles per gram of tissue.*

lel to the longitudinal axis of the stomach. Under the conditions employed in these experiments, such lesions occurred only in the corpus portion of the rat gastric mucosa.

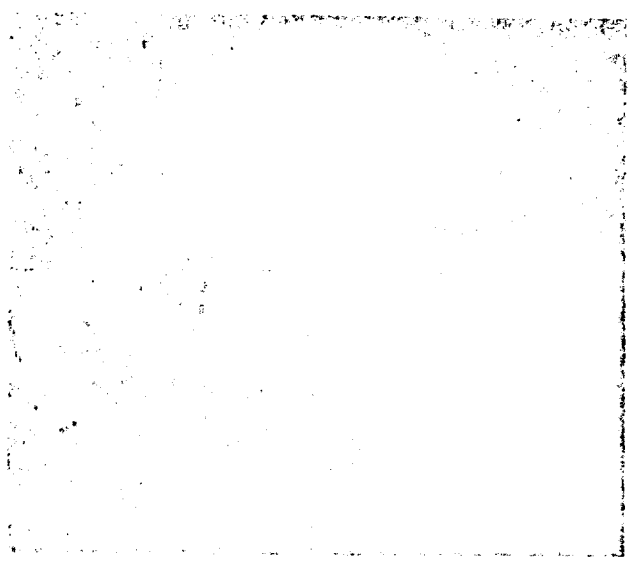

Figure 6—*Gross autoradiograph of mucosal surface of rat stomach 120 min. following oral administration of ¹⁴C-sodium benzoate.*

It may be seen in Fig. 2 that, of the compounds studied, only sodium acetylsalicylate caused gastric lesions in the rat. A mild erythema, rated as 2 on the scale, occurred in some cases after the administration of the other compounds. Formation of lesions was essentially maximal 60 min. after administration of sodium acetylsalicylate.

Concentration Study—The concentration in micromoles per gram of tissue of each ¹⁴C-labeled compound in gastric tissue was determined at each time interval. It is seen in Fig. 3 that the concentration of each compound in the corpus tissue diminishes rapidly with time.

The levels of these compounds in the rumen are remarkably different from the levels in the corpus (see Fig. 4). The concentration of sodium barbital in rumen tissue increases with time during the first 30 min. after its administration. Its concentration then drops

Figure 5—*Gross autoradiograph of mucosal surface of rat stomach 120 min. following oral administration of ¹⁴C-sodium acetylsalicylate.*

Figure 7—*Gross autoradiograph of mucosal surface of rat stomach 120 min. following oral administration of ¹⁴C-sodium barbital.*

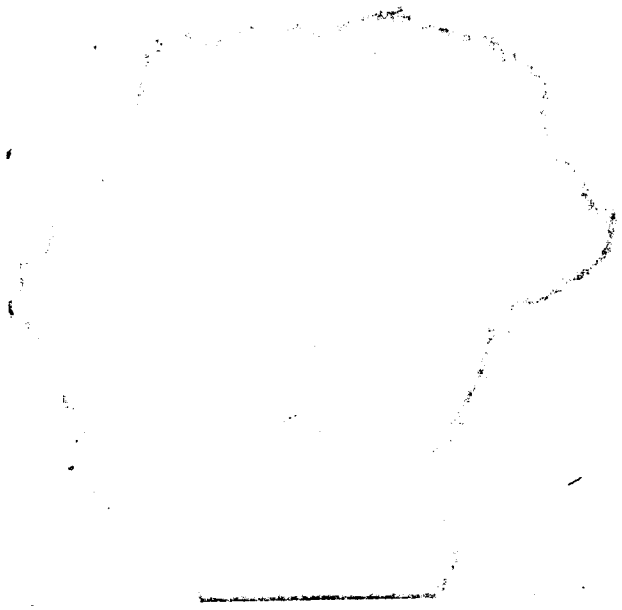

Figure 8 – *Gross autoradiograph of mucosal surface of rat stomach 5 min. following oral administration of ^{14}C-sodium acetate.*

for the remaining time intervals. The concentration of sodium acetylsalicylate diminishes very slowly throughout the entire period of observation. The concentration of sodium acetate in rumen tissue remains essentially constant for the first 30 min. after its administration and then drops rapidly in a manner similar to that of sodium barbital. Sodium benzoate levels decrease rapidly with time.

Distribution Study – Figures 5 through 10 show selected autoradiographs of the mucosal surfaces. All stomachs are positioned such that the rumen is at the top of the figure. The compounds are localized in the rumen of all stomachs up to 120 min. following administration with the exception of sodium acetate. Sodium acetate disappears from both the rumen and the corpus within 60 min. following administration (Figs. 5–10). Sodium acetylsalicylate, sodium benzoate, and sodium barbital demonstrate gastric distribution patterns similar to the pattern of acetylsalicylic acid reported by Morris *et al.* (5).

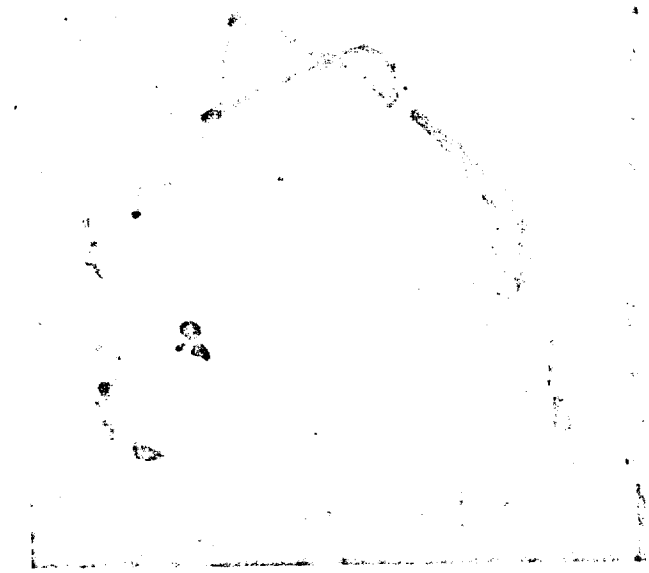

Figure 9 – *Gross autoradiograph of mucosal surface of rat stomach 60 min. following oral administration of ^{14}C-sodium acetate.*

Figure 10 – *Gross autoradiograph of mucosal surface of rat stomach, 120 min. following oral administration of ^{14}C-sodium acetate.*

SUMMARY AND CONCLUSIONS

The data presented in Fig. 2 indicate a high incidence of lesions following the administration of sodium acetylsalicylate. These findings are in agreement with those reported by Morris *et al.* (5) who used acetylsalicylic acid. Sodium acetylsalicylate, sodium benzoate, and sodium barbital showed similar localization patterns. It is obvious from both the quantitative data and the gross autoradiographs that these three compounds disappear with time from the corpus but remain in relatively high concentrations at all observed time intervals in the rumen portion. By contrast, the results of the autoradiographic localization and the tissue analysis of sodium acetate indicate a relatively rapid disappearance from both the rumen and corpus.

It should be emphasized that the concentration values reported represent a specific concentration at the designated time following administration, and that this value is a function of the penetration of the compound from the lumen into the tissue, possible metabolism, binding, absorption into the bloodstream, reabsorption from the blood into the stomach tissue, and possible loss of activity from the tissue into the lumen following tissue damage. Although the study does not provide an explanation of gastric ulceration by aspirin, the observations do not rule out the possibility that absorption characteristics of acetylsalicylic acid and its salts may be associated with their ability to cause gastric ulcers. Since sodium acetylsalicylate administration results in lesion formation and administration of the other compounds studied did not [illegible] in lesion formation, it becomes difficult to escape the con[illegible] that absorbed sodium acetylsalicylate interacts with the corpus [illegible] its secretions in some manner which is different from the [illegible] of the other weak acids. In addition, since lesions appear [illegible] in the corpus, through which sodium acetylsalicylate [illegible] and is absorbed, and not in the rumen, where the co[illegible] remains localized, it appears that the processes involved [illegible] tion and absorption play a role in the mechanism of lesion formation. Investigations into the nature of the interaction between sodium acetylsalicylate and corpus and rumen tissue are presently in progress.

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(1) M. J. H. Smith and P. K. Smith, "The Salicylates. A Critical Bibliographic Review," Interscience, New York, N. Y., 1966, p. 236.

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FACTORS AFFECTING THE METABOLISM OF ACETATE AND GLUCOSE BY RAM AND BULL SPERMATOZOA

By R. N. Murdoch* and I. G. White*

[*Manuscript received March 8, 1966*]

Summary

The potassium and magnesium concentrations of the suspending medium influenced the relative ease with which ram spermatozoa oxidized acetate and glucose.

In the absence of potassium or magnesium from the diluent acetate was usually oxidized at a lower rate than glucose and the oxygen uptake was correspondingly lower. The presence of 5–15 mM KCl stimulated the oxidation of acetate to a greater extent than that of glucose and in a medium containing potassium the rate of acetate oxidation usually approximated or exceeded that of glucose.

Potassium also stimulated anaerobic glycolysis and must affect the Embden-Meyerhof pathway as well as the Krebs-cycle reactions in ram spermatozoa.

While magnesium by itself did not influence the rate of oxidation of either acetate or glucose it suppressed the stimulatory effect of potassium on glucose metabolism. The use of a high phosphate (50 mM) diluent makes any differential effect of potassium on the oxidation of acetate and glucose difficult to observe since it depressed the oxidation of both substrates, presumably due to an effect on the tricarboxylic acid cycle.

Natural variability in the relative ease of oxidation of acetate and glucose was dependent on the potassium and magnesium levels in ram spermatozoa which are likely to vary from one ejaculate to another, with the extent of washing and the concentration of the cations in the suspending medium.

Bull spermatozoa always oxidized glucose more readily than acetate, irrespective of whether potassium and magnesium were added to the diluent. Bull spermatozoa would also seem to differ from ram spermatozoa in the fact that potassium stimulated glucose and acetate oxidation to approximately the same absolute amount. Furthermore, magnesium did not inhibit the stimulating effect potassium had on glucose oxidation.

I. Introduction

There is evidence that the pattern of glucose and acetate metabolism may differ in the spermatozoa of non-ruminant and ruminant species. Thus it would appear that boar, rabbit, fowl, and human spermatozoa oxidize glucose at a considerably faster rate than acetate, ram and bull spermatozoa oxidize acetate more readily than glucose or at a comparable rate (Terner 1960; Scott, White, and Annis 1963).

The latter response is, however, variable (Murdoch and White 1966). Some possible factors involved in the metabolism of glucose and acetate by ram and bull spermatozoa, namely washing and the cation and phosphate content of the medium, are examined in this paper. Previous studies of the effect of such treatment on the metabolism of spermatozoa have been reviewed by Salisbury and Lodge (1963).

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II. Materials and Methods

(a) *Semen*

Ram semen was collected by electro-ejaculation as described by Blackshaw (1951) and bull semen was collected with an artificial vagina. Only samples of good motility were used and care was taken to avoid sudden temperature changes during collection.

(b) *Diluents*

Two phosphate buffers (pH 7·0) were used:

(1) Low phosphate: 0·123M NaCl and 0·016M sodium phosphate (Umbreit, Burris, and Stanffer 1959) to which magnesium (0·001M $MgSO_4.7H_2O$) and potassium (0·005, 0·015, and 0·045M KCl) were added in place of corresponding concentrations of NaCl.

(2) High phosphate: 0·032M $NaH_2PO_4.H_2O$, 0·018M $Na_2HPO_4.12H_2O$, 0·036M NaCl (White 1953*a*). KCl (0·001 and 0·015M) was again added at the expense of the NaCl.

(1-^{14}C)Acetate and D-[U-^{14}C]glucose (from the Radiochemical Centre, Amersham, England) were added separately to the diluents to give concentrations of 54 μmoles (50 mc) and 18 μmoles (50 mc) per millilitre, respectively.

(c) *Preparation of Spermatozoal Suspensions*

Spermatozoa were washed by diluting the semen with 5 volumes of diluent and centrifuged for 10 min at 400 *g*. After aspirating the supernatant, the spermatozoa were washed again and diluted to the original volume with the diluent used for washing. For some experiments the washing procedure was repeated a third or fourth time.

(d) *Incubation of Spermatozoa*

Washed spermatozoal suspension (2 ml) was incubated at 37°C with 1 ml of substrate in 16-ml Warburg flasks containing CO_2-free 20% (w/v) KOH in the centre well. The final spermatozoal concentrations were $5·9 \times 10^8$/ml for the ram and $3·6 \times 10^8$/ml for the bull. Oxygen utilization was measured over 3 hr with air as gas phase and a shaking rate of 114 strokes/min. For experiments requiring anaerobic conditions, double-side-arm Warburg flasks containing 20% (w/v) KOH in the centre well, and yellow phosphorus and substrate in separate side arms, were gassed for 10 min with nitrogen. The stopcocks were then turned off and the substrate tipped into the spermatozoal suspension. Under aerobic conditions, acetate and glucose oxidation were determined from the assay of $^{14}CO_2$ trapped in the KOH of the centre well. Aliquots of the spermatozoal suspensions prior to incubation and of the flask contents after incubation were deproteinized by the addition of 0·5 ml of 5% (w/v) $ZnSO_4.7H_2O$ and 0·5 ml of 0·3N $Ba(OH)_2$ and glucose and lactate were estimated in the neutral filtrates. Counts of spermatozoa were made in duplicate and all values expressed on a 10^8 cell basis over the experimental period.

(e) *Analytical Method*

Lactate was determined by the enzymic method of Barker and Britton (1957) and glucose by the glucose oxidase method (Huggett and Nixon 1956).

(f) *Assay of Radioactivity*

The contents of the centre well were quantitatively removed by rinsing with CO_2-free water and the $^{13}CO_2$ absorbed was precipitated as $Ba^{13}CO_3$ in the presence of ammonium chloride. The $Ba^{13}CO_3$ was collected by filtration on Whatman No. 542 paper as described by Annison and White (1961) and assayed for radioactivity with an end-window Geiger Muller tube. The counts were corrected for self-absorption by the method of Hendler (1959).

(g) *Statistical Analysis*

All experiments were of a factorial design and the significance of the results has been assessed by analysis of variance. All main effects and their first-order interactions were isolated and tested for significance.

III. Results

(a) *Experiment 1*

The first experiment (Tables 1 and 2) was designed to study the metabolism of glucose and acetate by ram and bull spermatozoa after two and three washings in the low-phosphate diluent containing 0·001M magnesium and concentrations of potassium chloride varying from 0 to 45 mM.

(i) *Ram Spermatozoa.* The more severe washing significantly reduced the oxygen uptake and the substrate oxidized by ram spermatozoa whereas lactate production was not significantly reduced. Potassium, at all concentrations (5–45 mM), stimulated the oxidative metabolism of acetate; with glucose as substrate, 5–15 mM KCl produced no such stimulation and 45 mM was quite inhibitory.

(ii) *Bull Spermatozoa.* In contrast to ram spermatozoa, glucose was more effective than acetate in stimulating the oxygen uptake of bull spermatozoa and was also oxidized at a greater rate than acetate; the response did, however, vary with different ejaculates. Potassium was effective in increasing the oxygen uptake, substrate oxidation, and lactate production by the spermatozoa, 15–45 mM potassium being optimal. The stimulating effect of potassium in this instance occurred equally well with acetate or glucose as substrate.

(b) *Experiment 2*

This was similar to the first experiment except that no magnesium was added to the diluents (Tables 3 and 4).

(i) *Ram Spermatozoa.* Three as compared with two washings significantly reduced oxygen uptake and substrate oxidation as in the first experiment, and lactate production was not significantly reduced. Potassium at all concentrations increased the oxygen uptake, substrate oxidation and lactate production of the spermatozoa, the effects being somewhat greater after two rather than three washings. In the presence of potassium, acetate was again more effective than glucose in stimulating the oxygen uptake of the spermatozoa but in this experiment there was no [illegible] of glucose oxidation by the highest concentration of potassium (45 mM). Both the oxygen uptake and $^{13}CO_2$ readings show that acetate is oxidized less readily than glucose in the absence of potassium.

TABLE 1

EFFECT OF POTASSIUM ON THE METABOLISM OF [1-^{14}C]ACETATE AND D-[U-^{14}C]GLUCOSE BY RAM AND BULL SPERMATOZOA IN A VALUE CONTAINING MAGNESIUM

All values are calculated per 10^8 spermatozoa and represent the means of three ejaculates from each species. The low-phosphate diluent with equal magnesium present was used

Substrate	Potassium Concn. (mM)	Two Washings: Oxygen Uptake (µl)	Two Washings: Substrate Oxidized (µatoms C)	Two Washings: Lactate Produced (µmoles)	Three Washings: Oxygen Uptake (µl)	Three Washings: Substrate Oxidized (µatoms C)	Three Washings: Lactate Produced (µmoles)
Ram spermatozoa [1-^{14}C]Acetate	0	57·9	1·58	—	41·3	1·06	—
	5	70·0	1·62	—	67·1	1·68	—
	15	68·9	1·82	—	58·7	1·54	—
	45	77·2	1·88	—	60·8	1·52	—
D-[U-^{14}C]Glucose	0	53·4	1·92	1·96	48·4	1·68	2·22
	5	56·2	1·86	2·12	49·2	1·68	3·06
	15	56·9	1·92	2·78	44·6	1·50	2·49
	45	42·2	1·50	2·53	39·9	1·44	3·26
Bull spermatozoa [1-^{14}C]Acetate	0	12·1	0·32	—	12·4	0·34	—
	5	16·3	0·52	—	22·1	0·76	—
	15	24·7	0·72	—	21·8	0·70	—
	45	25·5	0·76	—	23·1	0·68	—
D-[U-^{14}C]Glucose	0	17·9	0·42	1·60	20·3	0·48	1·72
	5	18·8	0·48	1·92	28·5	0·78	2·74
	15	30·9	0·90	3·11	31·4	0·90	3·21
	45	39·7	0·96	3·80	27·4	0·78	3·84

TABLE 2

ANALYSIS OF VARIANCE OF THE DATA PRESENTED IN TABLE 1

The residual mean squares are given in italics at the base of the variance ratio columns

Source of Variation	Degrees of Freedom	Variance Ratios: Ram Spermatozoa, Oxygen Uptake	Ram Spermatozoa, Substrate Oxidized	Ram Spermatozoa, Lactate Produced	Bull Spermatozoa, Oxygen Uptake	Bull Spermatozoa, Substrate Oxidized	Bull Spermatozoa, Lactate Produced
Washings (A)	1	10·6**	11·8**	2·73	0·8	1·3	1·2
Substrates (B)	1	25·5**	2·0		15·6**	7·9**	
Potassium (C)	3						
Main effect (C_1)	1	5·5*	1·6	4·5	29·4**	45·6**	25·0**
Linear regression (C_2)	1	2·1	1·1	0·8	5·7*	5·4*	17·7**
Curvature (C_3)	1	0·04	0·4	0·14	2·7	2·2	0·1
Ejaculates (D)	2	20·2**	26·5**	12·59**	80·2**	88·3**	37·9**
First-order interactions							
A × B	1	0·8	0·2		0·6	0·0	
A × C	3	0·2	1·0	1·2	2·4	2·9	0·5
B × C	3						
B × C_1	1	10·2**	9·4**		0·13	0·04	
B × C_2	1	2·5	2·7		0·04	1·33	
B × C_3	1	1·6	0·3		0·76	1·13	
A × D	2	4·1*	2·5	1·0	0·9	2·1	0·9
B × D	2	2·5	1·0		4·6*	6·3**	
C × D	6	3·4	3·5	0·6	2·3	2·1	2·5
Residual	23	*92·80*	*0·06*	*0·37*†	*27·60*	*0·024*	*0·38*†

* $P < 0.05$ ** $P < 0.01$ † Six degrees of freedom.

TABLE 3

EFFECT OF POTASSIUM ON THE METABOLISM OF [1-^{14}C]ACETATE AND D-[U-^{14}C]GLUCOSE BY RAM AND BULL SPERMATOZOA IN A MAGNESIUM-FREE MEDIUM

All values are calculated per 10^8 spermatozoa and represent the means of three ejaculates from each species. The low-phosphate diluent with no added magnesium was used

Substrate	Potassium Concn. (mM)	Two Washings: Oxygen Uptake (μl)	Two Washings: Substrate Oxidized (μatoms C)	Two Washings: Lactate Produced (μmoles)	Three Washings: Oxygen Uptake (μl)	Three Washings: Substrate Oxidized (μatoms C)	Three Washings: Lactate Produced (μmoles)
Ram spermatozoa [1-^{14}C]Acetate	0	21·2	0·64	—	21·1	0·68	—
	5	66·4	1·90	—	61·4	1·66	—
	15	65·8	2·02	—	57·1	1·46	—
	45	71·4	1·94	—	65·2	1·78	—
D-[U-^{14}C]Glucose	0	31·8	1·02	1·05	32·2	1·02	0·85
	5	57·7	1·[illegible]	3·98	58·3	1·92	3·21
	15	63·0	2·[illegible]	6·06	46·9	1·74	3·87
	45	56·0	1·[illegible]	5·94	50·3	1·62	5·11
Bull spermatozoa [1-^{14}C]Acetate	0	9·4	0·[illegible]	—	7·0	0·18	—
	5	16·6	0·[illegible]	—	13·2	0·40	—
	15	16·3	0·56	—	14·4	0·43	—
	45	18·0	0·[illegible]	—	14·1	[illegible]	
D-[U-^{14}C]Glucose	0	12·7	[illegible]	1·61	11·6	0·30	2·[illegible]
	5	20·5	0·60	2·26	16·7	0·48	2·[illegible]
	15	22·4	0·[illegible]	2·13	18·9	[illegible]	2·73
	45	22·2	0·66	3·54	18·7	[illegible]	3·17

TABLE 4

ANALYSIS OF VARIANCE OF THE DATA PRESENTED IN TABLE 3

The residual mean squares are given in italics at the base of the variance ratio columns

Source of Variation	Degrees of Freedom	Variance Ratios: Ram Spermatozoa: Oxygen Uptake	Ram Spermatozoa: Substrate Oxidized	Ram Spermatozoa: Lactate Produced	Bull Spermatozoa: Oxygen Uptake	Bull Spermatozoa: Substrate Oxidized	Bull Spermatozoa: Lactate Produced
Washings (A)	1	12·0**	14·5**	3·6	65·0**	3·0	0·08
Substrates (B)	1	8·2**	8·0**		122·8**	8·0	
Potassium (C)	(3)						
Main effect (C_1)	1	372·1**	226·5**	37·8**	287·6**	86·0**	30·7**
Linear regression (C_2)	1	0·0	0·3	6·7*	8·3**	9·0**	31·6**
Curvature (C_3)	1	2·0	0·8	0·4	2·1	0·1	0·04
Ejaculates (D)	2	38·8**	37·0**	10·5*	66·1**	37·0**	20·9**
First-order interactions							
A × B	1	0·0	0·0		0·0	1·0	
A × C	3	3·1*	4·3*	0·6	1·1	1·3	1·3
B × C	(3)						
B × C_1	1	33·8**	3·5		0·9	0·3	
B × C_2	1	5·0*	2·5		0·2	0·4	
B × C_3	1	1·2	2·0		1·7	1·8	
A × D	2	10·4**	9·3**	2·3	8·6**	0·5	0·5
B × D	2	0·3	1·0		22·2**	11·0**	
C × D	6	19·9**	9·0**	3·0	4·3**	2·8**	0·2
Residual	23	27·1	0·04	1·67*	1·8	0·01	0·13†

* $P < 0·05$. † $P < 0·01$. ‡ Six degrees of freedom.

(ii) *Bull Spermatozoa.* More severe washing in this experiment reduced the oxygen uptake of bull spermatozoa below that of spermatozoa washed twice. Glucose was again more effective than acetate in stimulating the oxygen uptake of the spermatozoa. Potassium stimulated all metabolic parameters for both substrates and again the higher levels of potassium (15–45 mM) were more effective than 5 mM potassium.

TABLE 5

EFFECT OF MAGNESIUM AND POTASSIUM ON THE METABOLISM OF D-[U-^{14}C]GLUCOSE BY TWICE-WASHED RAM SPERMATOZOA

Values are calculated per 10^9 spermatozoa and represent the means of four ejaculates. The low-phosphate diluent was used. The residual mean squares are given in italics at the base of the variance ratio columns.

Magnesium Concn. (mM)	Potassium Concn. (mM)	Oxygen Uptake (μl)	Glucose Oxidized (μatoms C)	Lactate Produced (μmoles)
0	0	42·3	1·19	2·21
0	15	50·9	1·50	2·83
1	0	43·6	1·20	2·08
1	15	47·0	1·19	2·77

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio: Oxygen Uptake	Variance Ratio: Substrate Oxidation	Variance Ratio: Lactate Produced
Magnesium (A)	1	0·12	26·7*	0·44
Potassium (B)	1	2·17	26·7*	19·00*
Ejaculates (C)	3	3·03	46·7**	12·22*
First-order interaction				
A × B	1	0·39	36·7**	0·60
A × C	3	0·80	1·0	1·22
B × C	3	0·17	4·3	7·33
Residual	3	*69*	*0·003*	*0·09*

* $P < 0·05$. ** $P < 0·01$.

(c) *Experiment 3*

A comparison of the results from experiments 1 and 2 suggested that magnesium might depress the stimulatory effect of potassium on glucose metabolism and the possibility of such an interaction was tested in this experiment using D-[U-^{14}C]glucose and twice-washed ram spermatozoa suspended in the low-phosphate diluent.

Table 5 shows that in the absence of magnesium, potassium increased the oxidation of glucose, oxygen uptake, and accumulation of lactate. The presence of

0·001 M magnesium, however, inhibited the stimulatory effect of potassium on oxidative metabolism. Magnesium, in the absence of potassium, had no effect on the metabolism of glucose.

(d) *Experiment 4*

Since a low phosphate buffer had been used in the previous studies and since the phosphate content of the medium may influence metabolism, the next experiment (Table 6) was designed to study the effect of potassium (4 mM) on glucose and acetate metabolism by ram spermatozoa washed four times in high-phosphate buffer. Glucose

TABLE 6

EFFECT OF POTASSIUM IONS IN HIGH-PHOSPHATE BUFFER ON 0·4 [U-^{14}C]GLUCOSE AND [1-^{14}C]ACETATE METABOLISM BY RAM SPERMATOZOA WASHED FOUR TIMES

Values are calculated per 10^9 spermatozoa and represent the means of four ejaculates. The residual mean squares are given in italics at the base of the variance ratio columns

Substrate	Potassium Concn. (mM)	Oxygen Uptake (μl)	Substrate Oxidized (μatoms C)	Lactate Produced (μmoles)
[1-^{14}C]Acetate	0	22·8	1·02	
	4	28·6	1·20	—
[U-^{14}C]Glucose	0	29·6	1·14	0·79
	4	36·5	1·50	1·13

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios		
		Oxygen Uptake	Substrate Oxidization	Lactate Produced
Substrate (A)	1	63·0**	50·0**	
Potassium (B)	1	51·2**	86·7**	1·6
Ejaculates (C)	3	10·2**	71·0**	7·0
First order interactions				
A × B	1	0·9	6·7	
A × C	3	1·3	0·0	
B × C	3	13·9**	53·3**	
Residual	3	*3·2*	*0·003*	*0·6*

** $P < 0·01$

more effective than acetate in stimulating the oxygen uptake of ram spermatozoa when the two substrates and was oxidized at a greater rate. Potassium stimulated the oxygen uptake and oxidation of both substrates and the accumulation of lactate from glucose.

(e) *Experiment 5*

Since potassium stimulated rather than depressed oxygen uptake in the previous experiments it seemed likely that the increased aerobic accumulation of lactate was due to a direct stimulation of the Embden–Meyerhof pathway; however, the point

was proved by studying the effect of potassium on the anaerobic glycolysis of ram spermatozoa washed twice in the low-phosphate diluent. Table 7 shows that 15 mM potassium significantly stimulated the anaerobic production of lactate from glucose.

TABLE 7

EFFECT OF POTASSIUM IONS ON THE ANAEROBIC METABOLISM OF GLUCOSE BY RAM SPERMATOZOA

Values are calculated per 10^9 spermatozoa and represent the means of four ejaculates. The low-phosphate diluent with no added magnesium was used. The residual mean squares are given in italics at the base of the variance ratio columns

Potassium Concn. (mM)	Glucose Utilization (μmoles)	Lactate Production (μmoles)
0	1·61	3·78
15	2·56	6·33

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios	
		Glucose Utilization	Lactate Production
Potassium	1	48·92**	22·05*
Ejaculates	3	3·78	3·14
Residual	3	*0·037*	*0·59*

* $P < 0·05$. ** $P < 0·01$.

(f) *Experiment 6*

In a final experiment the effect of potassium on the metabolism of acetate, glucose, and lactate was compared when ram spermatozoa were washed and suspended in high- and low-phosphate buffers. All criteria show (Tables 8 and 9) that the metabolism of the three substrates was depressed by high phosphate buffer and the stimulatory effect of potassium on oxidation and glycolysis was much less in such a medium.

IV. DISCUSSION

The present study confirms previous reports that low concentrations of potassium stimulate the oxidative metabolism and aerobic glycolysis of glucose or fructose in spermatozoa (Lardy and Phillips 1943; White 1953*b*; Wallace and Wales 1964). Since, however, we have also shown that potassium stimulates anaerobic glycolysis as well as the oxidation of acetate and lactate, potassium must have a stimulatory effect on both the Embden–Meyerhof pathway and the Krebs-cycle reactions in spermatozoa. Boyer, Lardy, and Phillips (1942, 1943) have shown that

TABLE 8

[illegible] ACETATE, GLUCOSE AND [illegible] BY TAP-WASHED RAM SPERMATOZOA

Values are mean [illegible] per [illegible] for [illegible] of three replicates

Substrate	Potassium (mM)	Oxygen Uptake (µl)	Substrate Oxidized (atoms C)	Lactate Produced (µmoles)	Lactate Utilized (µmoles)	Oxygen Uptake (µl)	Substrate Oxidized (atoms C)	Lactate Produced (µmoles)	Lactate Utilized (µmoles)
		High phosphate buffer				Low phosphate buffer			
Glucose	0	24·7	0·47			25·3	0·74*		
	15	33·1	1·05			68·0	1·73		
Glucose + acetate	0	26·7	0·34	0·62		40·4	1·20	1·26	
	15	32·3	0·92	1·05		59·3	1·64	2·12	
Lactate	0	23·3	0·65		0·53	38·3	0·78		0·58
	15	26·4	0·33		0·41	67·7	1·20		0·80

potassium is necessary for the conversion of phosphopyruvate to pyruvate by muscle extracts and Muntz (1947) found that it also participates in the phosphokinase reaction of yeast juice. Both reactions are involved in the glycolytic cycle and might be the point at which potassium affects ram and bull spermatozoa.

Potassium is known to stimulate the aerobic metabolism of brain (Ashford and Dixon 1935; Lipsett and Crescitelli 1950) and although its site of action in the Krebs cycle of reactions has not been pinpointed it may indirectly increase the availability of phosphate-acceptor ADP and inorganic phosphate. At all events the critical effect of potassium on the activity of ram spermatozoa appears to be related to their ability to produce or make use of energy from the oxidation of exogenous substrate over the Krebs cycle (Dott and White 1964).

TABLE 9

ANALYSIS OF VARIANCE OF THE DATA PRESENTED IN TABLE 8

The residual mean squares are given in italics at the base of the variance ratio columns

Source of Variation	Degrees of Freedom	Variance Ratios		
		Oxygen Uptake	Substrate Oxidized	Lactate Produced
Buffer (A)	1	112·9**	60·00**	35·25*
Substrates (B)	(2)			
Acetate *v.* lactate (B_1)	1	0·5	9·67**	
Glucose *v.* rest (B_2)	1	1·9	7·67*	
Potassium (C)	1	64·0**	51·00**	91·50*
Ejaculates (D)*	2	21·8**	13·00**	0·75
First order interactions				
$A \times B$	(2)			
$A \times B_1$	1	2·6	0·03	
$A \times B_2$	1	0·004	5·33*	
$A \times C$	1	23·3**	11·33**	0·00
$B \times C$	(2)			
$B_1 \times C$	1	3·7	10·67**	
$B_2 \times C$	1	6·8*	1·00	
$A \times D$	2	0·8	2·50	2·75
$B \times D$	4	0·3	0·23	
$C \times D$	2	0·6	2·83	5·00
Residual	16	*43·6*	*0·03*	*0·01*†

* $P < 0·05$. ** $P < 0·01$. † Two degrees of freedom.

Cragle and Salisbury (1959) have reported that very high concentrations of potassium depress the metabolism of bull spermatozoa. There was no indication in our studies that potassium by itself was toxic, although in the presence of 0·001M magnesium, 15 mM potassium did decrease the ability of ram spermatozoa to oxidize glucose. Magnesium has a direct effect on the formation of succinic acid from 2-oxoglutaric acid in the tricarboxylic acid cycle (Krebs and Lowenstein 1960) and this could possibly be the site of the interaction between magnesium and potassium.

Wales and O'Shea (1966) have also found that the addition of potassium to ram spermatozoa increases the oxidation of acetate to a greater extent than that of fructose. It is tempting to suggest that potassium facilitates a more ready combination of acetate with CoA than is the case with pyruvate, a breakdown product of glucose via the Embden Meyerhof pathway and an alternative precursor of acetyl-CoA. Other workers have reported that potassium increases the rate of entry of acetate into the tricarboxylic acid cycle (Beinert *et al.* 1953).

The present studies confirm the depressing effect of high concentrations of phosphate (80 mM) on the oxidative metabolism of ram spermatozoa reported by Mann and White (1956). Ram spermatozoa are, however, much less susceptible to phosphate than bull spermatozoa; thus concentrations of up to 60 mM apparently stimulate the metabolism of ram spermatozoa (Wallace and Wales 1964) but as little as 6 mM depresses the respiration of washed bull spermatozoa (Lodge *et al.* 1963; Wales and Wallace 1964). Since the oxidation of acetate is depressed to the same extent as that of glucose or lactate (Table 8) the rate-limiting action of phosphate, as far as oxygen uptake is concerned, must be concerned with the tricarboxylic acid cycle although aerobic glycolysis may to some extent be also reduced. This suggestion is supported by the observation that potassium was almost ineffective in stimulating the oxidation of glucose by ram spermatozoa in the high-phosphate buffer although aerobic glycolysis was greatly improved.

It is clear from these experiments that the potassium and magnesium concentrations of the diluent will influence the relative ease with which ram spermatozoa oxidize acetate and glucose. In the absence of potassium or magnesium from the diluent acetate was usually oxidized at a lower rate than glucose and the oxygen uptake was correspondingly lower. The presence of 5–15 mM KCl stimulated the oxidation of acetate to a greater extent than that of glucose and in a medium containing potassium the rate of acetate oxidation usually approximated or exceeded that of glucose. While magnesium in itself does not influence the rate of oxidation of either acetate or glucose it suppresses the stimulatory effect of potassium on glucose metabolism so that in a medium containing a fairly high concentration of potassium (5–15 mM) and magnesium (0·001M), ram spermatozoa show the highest rate of acetate oxidation relative to glucose. The use of a high-phosphate diluent makes any [illegible] effect of potassium on the oxidation of acetate and glucose difficult to [illegible] since it depresses the oxidation of both substrates.

These results suggest that the variability in the relative ease of oxidation of [illegible] and glucose may depend on the potassium and possibly also the magnesium [illegible] in the spermatozoa. These vary from one ejaculate to another and also with the [illegible] of the bull and the concentration of the cations in the suspending medium (Quinn, White, and Wittick, 1965, 1966).

In the experiments reported here bull spermatozoa always oxidized glucose more readily than acetate irrespective of whether potassium and magnesium were [illegible] in the diluent. Bull spermatozoa would also seem to differ from ram spermatozoa in the fact that potassium stimulates glucose and acetate oxidation to about the same absolute amount. Furthermore magnesium does not inhibit the stimulating effect of potassium on glucose oxidation as is the case with ram spermatozoa.

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The effect of histamine combined with formic or acetic acid on food intake and rumen motility, when infused into the omasum of a ram*

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SUMMARY

After establishing a permanent omasal fistula in a ram, solutions containing histamine with or without formic or acetic acid were introduced directly into the omasum. It was found that histamine, together with formic or acetic acid, stopped feeding and rumen motility, but formic acid and histamine, infused separately, had no effect on food intake.

In a recent paper Neumark (1967) showed that solutions containing histamine and formic acid strongly depressed the food intake of sheep, when introduced via a stomach tube to a certain depth in the rumen.

The experimental findings indicated that some part of the stomach, other than the rumen, must be the site of action of these compounds and the omasum was suggested as sensitive to these compounds.

The experiments reported here were designed to locate the exact site of action of these compounds in the sheep stomach. It was found that histamine combined with formic or acetic acid, when infused into the omasum, depressed food intake whereas each compound infused separately had no effect.

MATERIALS AND METHODS

A Merino ram weighing 170 lb was fitted with an omasal fistula, according to the method of Willes & Mendel (1964). The animal recovered rapidly from the operation and 3 weeks later was accustomed to a fixed feeding programme. The animal was fed 350 g chopped hay at 08.30 a.m. and 04.00 p.m.; after the hay was finished an additional 200 g concentrates was given.

Feeding speed and behaviour were measured and the term 'fed normally' is used to signify that the ram fed for at least 18 min (the average feeding time, as calculated from 20 measurements, was 20 min), and fed almost continuously during this time, making no more than four short interruptions. 'Slightly depressed' signifies that the ram made many interruptions of longer duration during the feeding period. 'Stopped feeding' means that the animal fed for only a very short period (5–8 min), lay down afterwards, and resumed feeding only after half an hour or more.

On the days of experiment the infusions (generally of 500 c.c. in volume) were made at about 08.30 a.m. The infusion was completed within 4 min. In all trials histamine was given as di-HCl-histamine and analar grade formic acid was used.

Treatments 1–6 (Table 1) were designed to determine the lowest level of formic acid, infused with a constant amount of histamine, at which food intake was depressed. Treatments 1–4 were carried out in duplicate and treatment 5 in a triplicate. In treatments 7–9 (Table 1) histamine or formic acid was infused separately in order to investigate the effect of each of the two compounds *per se*. Treatment 10 was designed to investigate whether, after replacing formic acid by its sodium salt, histamine would exert the same effect upon food intake as with formic acid.

In treatments 11 and 12 (Table 1) formic acid was replaced by hydrochloric acid in order to find out if the acidity *per se* of the solution containing histamine was sufficient to depress appetite. Treatments 13 and 4 (Tables 1 and 2 respectively) were designed to determine whether acetic acid had the same effect as formic acid, or if the effect of formic acid was specific. In treatments 14–16 (Table 1) histamine was replaced by its metabolic precursor, histidine, and by amines such as tryptamine or serotonine, often present in silages (Neumark, Bondi & Volcani, 1964), in order to discover if the effect of histamine was specific.

The treatments, summarized in Table 2, were similar to some of those presented in Table 1, but in addition to the influence upon food intake the

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Table 1. *Response of the omasal fistulated ram to different solutions introduced directly into the omasum*

	Treatment*		Response
	Formic acid (c.c.)	Histamine† (mg)	
1.	3·0	214	Stopped feeding
2.	4·2	405	
3.	3·0	400	
4.	2·0	400	
5.	1·0	400	
6.	0·5	400	Fed normally
7.	None	None	
8.	3·0	None	
9.	3·5		
	Sodium formate (g)	Histamine (mg)	
10.	8·0	406	Fed normally
	6 N-HCl/c.c.	Histamine/mg	
11.	0·9	403	Fed normally
12.	1·0	600	
	Acetic acid glacial (c.c.)	Histamine (mg)	
13.	6·4	492	Food intake slightly depressed
	Formic acid/c.c.	Histidine/mg	
14.	3·5	500	Fed normally
	Formic acid/c.c.	Tryptamine/mg	
15.	3·5	450	Fed normally
	Formic acid/c.c.	Serotonine/mg	
16.	3·0	400	Fed normally

* In all trials except treatment 1, the compounds introduced were dissolved in 500 c.c. distilled water, in treatment 1 only 100 c.c. were used.

† Histamine is calculated as di-HCl-Histamine. Tryptamine was administered as HCl-tryptamine, and serotonine as serotonine-creatinine-sulphate.

Table 2. *Introduction of different solutions (500 c.c.) into the omasum and their effect on rumen motility*

	Treatment			Response
	Formic acid/c.c.	Histamine/mg	pH	
1.	3·5	407	2·1	No rumen motility; stopped feeding
2.	3·5	None	2·1	Rumen motility normal; feed normally
3.	3·5	400	5·4*	
	Acetic acid/c.c.			
4.	8·0	500	2·6	No rumen motility; stopped feeding

* Formic acid and histamine dissolved in 500 c.c. sodium phosphate buffer at pH 5·4.

effect upon rumen motility was also checked. The rumen motility was measured with the help of a stethoscope for listening to and counting the sounds made by the ruminal contractions. The rumen motility was measured for 5 min, both before and after treatment. There were normally 7 or 8 contractions during a 5-min period. The measurement of rumen motility after treatment started about 7–8 min after completion of the infusion.

RESULTS

From treatments 1–5 (Table 1) it can be seen that formic acid together with histamine strongly depressed the food intake. When the amount of histamine was kept constant (400 mg), the lowest effective concentration of formic acid was found to be 1 c.c. per 500 c.c. water (treatment 5). Treat

ments 7–9 (Table 1) show that neither histamine nor formic acid had any effect upon food intake when infused separately into the omasum. From treatment 10 (Table 1) it can be seen that formate given as sodium formate together with histamine had no influence upon the feeding of the ram, although in this trial the amount of the formate ion was greater (on a molar basis) than the amount of formate ion, contained in 4·2 c.c. formic acid, given in treatment 2 (Table 1). The same result was obtained in treatment 3 (Table 2), when formic acid was dissolved in a sodium phosphate buffer at pH 5·4.

When 6·4 c.c. acetic acid replaced the formic acid, the food intake of the ram was slightly depressed (the animal made many interruptions during the feeding period). Raising the amount of acetic acid to 8·0 c.c. but keeping the histamine level approximately constant caused the ram to stop feeding in treatment 4 (Table 2). When histamine was replaced by the amino acid histidine or by amines such as tryptamine or serotonine (treatments 14, 15 and 16 respectively, Table 1), the normal feeding behaviour of the ram was not affected.

The observations on rumen motility are summarized in Table 2, which shows that where infusion caused an interruption of feeding there was cessation in rumen motility, but where the infusion had no effect on feed intake there was no change in rumen motility.

DISCUSSION

The present results lend confirmation to the previous conclusions of Neumark (1967) that the site of action of histamine and formic acid is post ruminal. Although it was not possible in this experiment to prove directly (e.g. the ligature of the omasal abomasal orifice and subsequent infusion of the solution) that the omasum is the site of action of histamine and formic acid, the following experimental findings seem to justify the conclusion that only the omasum or some parts of it are sensitive to these compounds.

A. In a previous paper Neumark (1967) showed that solutions of formic acid and histamine infused directly into the pyloric region of the abomasum of sheep had no influence upon feed intake.

B. From treatments 10 and 3 (Table 1 and 2) it can be seen that when the formate was present in the solutions as its sodium salt, no feed-depressing effect was obtained.

The experimental results, cited in A, make it improbable that the abomasum could be the site of action for histamine and formic acid.

The assumption that any part of the intestine could be the site of action is difficult to maintain for the following reasons: according to Ash (1961) the pH of abomasal contents in sheep is 2·2–2·5 with very small fluctuations, as the abomasum of ruminants secretes hydrochloric acid continuously. Therefore most, if not all, of the sodium formate infused in treatment 10 must be converted to formic acid on entering the abomasum (pH 2·2–2·5). When formic acid was added to a sodium phosphate buffer solution (pH 5·4, treatment 3, Table 2), the acid secretion of the abomasum must have been stimulated since according to Ash (1959), phosphate buffers (pH 5–6) containing 25 or 50 mM fatty acids, when introduced into the abomasum, produce larger acid secretion of the abomasum than phosphate buffer alone. From this statement the conclusion can be drawn that the introduction into the omasum of the formate as sodium formate, formic acid or formic acid buffered at pH 5·4 cannot have any influence on the final chemical state of the formate in the abomasum (i.e. undissociated formic acid), and virtually undissociated formic acid leaves the abomasum and enters the intestine. It therefore seems justified to exclude the intestine as a possible site of action for solutions containing formic acid and histamine, as only formic acid with its low pH and histamine introduced into the omasum depressed the feed intake, whereas histamine and the slightly basic sodium formate or formic acid buffered at pH 5·4 and introduced into the omasum (treatments 10 and 3, Tables 1 and 2 respectively) did not affect the feed intake of the ram. Treatment 4 (Table 2), where acetic acid replaced formic acid, is of special interest. Acetic acid represents the major part of the volatile fatty acids in sheep rumen (Hungate, 1966). A considerable proportion of the acids reach the omasum, where most of them are absorbed (Hungate, 1966) and some of them are metabolized by the omasal epithelium (Johnston, Kesler & McCarthy, 1961; Joyner, Kesler & Holter, 1963). In the light of these findings the assumption seems to be justified that when histamine is introduced into the rumen (e.g. by certain types of silages) or formed in significant amounts by the rumen microbes, this amine may interfere with food intake. It appears that the site of action of histamine in association with organic acids is not in the rumen but in the omasum or some part of it. However, the observations on cessation of rumen motility indicate that histamine may have an effect on the rumen *per se* and may be involved in causing bloat in ruminants (Dougherty, 1943). Furthermore, the fact that infusion of the above solutions into the omasum depressed the motility of the rumen indicates a systemic effect.

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CHOLESTEROL METABOLISM AND THE BLOOD-BRAIN BARRIER: AN EXPERIMENTAL STUDY WITH 2-C^{14} SODIUM ACETATE

BY

HAROLD J. NICHOLAS AND BYRON E. THOMAS

When deuterium-labelled water or acetic acid was presented to adult rats (Waelsch, Sperry and Stoyanoff, 1940*a*; Bloch, 1950) little or no deuterium was found in cholesterol isolated from the brains of these animals. In addition, it has been the uniform experience of investigators studying cholesterol metabolism that adult brain reluctantly, if at all, incorporates steroid precursors into adult brain cholesterol *in vitro* (Srere *et al.*, 1950; Azarnoff, Curran and Williamson, 1958). In contrast, new born and young brain readily incorporated labelled precursors into the cholesterol molecule *in vitro* (Srere *et al.*, 1950) or *in vivo* (Waelsch, Sperry and Stoyanoff, 1940*b*). Based on this type of experimentation it has been concluded that as an animal reaches maturity the brain loses or almost completely loses its capacity to metabolize cholesterol. Recently, however, two different laboratories have shown that when C^{14} labelled sodium acetate or 2-C^{14}- sodium mevalonate was injected intracerebrally (Nicholas and Thomas, 1959*a*, *b*) or intracisternally (McMillan, Douglas and Mortensen, 1957) into adult rats, presumably by-passing the blood-brain barrier, considerable C^{14} was found in brain cholesterol. A complete evaluation of these various data cannot be made at this time. A reasonable interpretation of the intracerebral data, however, would appear to be that adult rat brain tissue does possess an inherent capacity to synthesize cholesterol[1] but that this capacity is inhibited or held in check by factors at present largely unknown. By inference with a vast amount of information in adjacent areas of study (Bakay, 1956) the blood-brain-barrier seems a logical choice as one of the controlling factors. On this account it has seemed pertinent to investigate in more detail the effect of the blood-brain-barrier on brain cholesterol metabolism

[1] It should be recalled that *in vitro* experiments do not measure "turnover," and that unless one assumes a net increase of cholesterol during a short period following intracerebral injection of the precursors, some concurrent turnover of cholesterol must have occurred during this short period. It seems premature to discuss this point in more detail at this time.

since this "barrier" may control not only the influx of cholesterol precursors to the brain but the metabolism of cholesterol within the brain as well.

Several aspects related to this problem were investigated for this manuscript. First, if sodium acetate does not penetrate into the adult brain as a result of the barrier, then where the barrier is non-existent or of limited effect (as in fœtal or very young rats) acetate should penetrate into the brain and become incorporated into the cholesterol molecule. Strangely enough, there is no information in the literature on this question. Secondly, since the adult brain seems reluctant to incorporate acetic acid given parenterally or *in vitro* but does so when the precursor is injected directly into the brain, it has occurred to us that the cholesterol biosynthesis demonstrated by intracerebral injection may not be a true tissue biosynthesis but rather the result of secondary reactions occurring because of the brain wound. Tissue reactions in response to stab wounds have been explored in some detail (Macklin and Macklin, 1920; Hess, 1955*a*). The effect of a massive dose of labelled sodium acetate injected intraperitoneally into a normal adult rat was investigated also. The rationale for this experiment was the possibility that competition of other tissue sources (e.g. the liver) for the acetate may limit the amount of acetate reaching the brain when injected systemically. Lastly, while this work was in progress some observations on mouse brain cholesterol metabolism appeared (Kabara and Okita, 1959) and we have added our own observations obtained on this species.

MATERIALS AND METHODS

$C^{14}H_3COONa$ was used as the cholesterol precursor in these experiments. It had a specific activity of 6·02 mcmM and was purchased from Isotopes Specialties, Inc., Burbank, California. Intracerebral injections were performed as previously described (Nicholas and Thomas, 1959*a*). Rats and mice were of the Sprague-Dawley and CFW strain respectively, and guinea-pigs were purchased from a local dealer. All animals were sacrificed by decapitation. Where cholesterol concentration was to be determined, livers and brains were rinsed in distilled water and dried to constant weight *in vacuo*. The dried tissue was then saponified and the non-saponifiable fraction (obtained as previously outlined: Nicholas and Thomas, 1959*a*) was dissolved quantitatively in acetone-absolute ethanol (1:1). Duplicate or triplicate aliquots were then taken for cholesterol determination according to the Sperry-Webb procedure (Sperry and Webb, 1950). The remaining solution was used to obtain cholesterol digitonide, which was plated and counted for C^{14} activity according to earlier description (Nicholas and Thomas, 1959*a*). Fatty acids, where investigated, were also obtained and counted as previously described. The digitonides were not further treated, since it has been our experience with *in vivo* work of this nature that counts obtained on brain cholesterol digitonides correlate well with counts obtained from corresponding samples converted to the dibromides via the free sterol (i.e., the radioactivity in the digitonides is due largely to C^{14}-labelled cholesterol). In some of the experiments free cholesterol was obtained from the digitonide by the pyridine method and counted directly, after plating on 2·5 cm. diameter nickel planchets at a thickness of 1 mg. per planchet. Where a crude non-saponifiable fraction was investigated the material was similarly plated at 1 mg. per planchet. In those experiments in which a crude squalene fraction was investigated the general chromatographic separation of Langdon and Bloch (1953) was

used, the squalene being removed from Fisher chromatographic grade alumina (80–200 mesh) with petroleum ether (b.p. 30–60°), and a crude cholesterol fraction subsequently eluted with acetone-ethyl ether (1:1).

A pregnant rat and guinea-pig respectively were each injected with $5{\cdot}0 \times 10^7$ c.p.m. of $C^{14}H_3COONa$ several days before parturition. The rat delivered one young three days later (found on entering the laboratory in the morning) and was thereupon sacrificed under ether anæsthesia. Four more rats were removed *in utero* and sacrificed immediately. The pregnant guinea-pig was sacrificed twenty-four hours after injection with three hairless guinea-pigs removed *in utero*.

Two six-month-old female rats were subjected to intracerebral injection of 0·1 ml. of distilled water only, into the left cerebral hemisphere. Each rat thereupon received $1{\cdot}0 \times 10^7$ c.p.m. of $C^{14}H_3COONa$ intraperitoneally within ten minutes; they were sacrificed two hours after injection of the acetate. The brain was immediately removed and saponified; free cholesterol was obtained through the digitonide and counted directly. Fatty acids were also obtained and counted. In another series, 4 discarded breeder rats were similarly injected intracerebrally with 0·1 ml. of distilled water. Ten minutes later each was injected intraperitoneally with $4{\cdot}4 \times 10^7$ c.p.m. $C^{14}H_3COONa$. They were subsequently sacrificed at intervals of ten and twenty minutes, and one and three hours respectively. The brains were immediately saponified and non-saponifiable and fatty acid fractions obtained.

To determine whether a rather large dose of intraperitoneally administered sodium acetate would more effectively penetrate brain tissue a 340 gramme discarded breeder rat was injected intraperitoneally with $C^{14}H_3COONa$ at a dosage of 500 microcuries per kilogram of weight. Sacrifice was made ten minutes later, at which time the liver and brain were immediately saponified. Crude squalene and cholesterol fractions were obtained as described by Nicholas and Thomas (1959*b*) and fatty acids as described previously.

TABLE I.—INCORPORATION OF $C^{14}H_3COONa$ INTO CHOLESTEROL OF IMMATURE RATS; INTRACEREBRAL VS INTRAPERITONEAL INJECTION*

		Cholesterol specific activity†			
		Brain		*Liver*	
Rat No.	*Time sacrificed after injection (hrs.)*	*c.p.m. per mg.*	*c.p.m. per mg. chol. per gramme dried tissue*	*c.p.m. per mg.*	*c.p.m. per mg. chol. per gramme dried tissue*
1	2	763	22,890	105	1,124
2	2	680	18,000	—	—
3	2	128	3,878	92	902
4	2	111	3,430	274	2,713
5	4	119	3,737	—	—
6	4	67	2,050	134	1,541
7	4	129	4,154	—	—
8	6	172	5,470	—	—
9	6	172	3,732	—	—
10	6	99	3,523	—	—
11	24	171	6,047	—	—
12	24	121	5,265	—	—

*All rats were ten days old and weighed 20–23 grammes. Each received $5{\cdot}0 \times 10^6$ c.p.m. $C^{14}H_3COONa$, rats No. 1 and 2 by intracerebral injection, the others intraperitoneally.

†Calculated from infinitely thick samples of cholesterol digitonide.

RESULTS

The blood-brain barrier present in fœtal and young guinea-pigs (Hess, 1955*b*) and more recently shown to be present for some dyes even in neonatal and very young rats (Millen and Hess, 1958) was not effective against sodium acetate in tracer doses. Labelled acetate given intraperitoneally to immature rats and guinea-pigs (Tables I and II) was found

TABLE II.—INCORPORATION OF $CH^{14}C_2OONa$ INTO NEWBORN AND YOUNG GUINEA-PIGS; INTRACEREBRAL VS INTRAPERITONEAL INJECTION*

		Cholesterol specific activity†			
		Brain		*Liver*	
Guinea-pig No.	*Time sacrificed after injection (hrs.)*	*c.p.m. per mg.*	*c.p.m. per mg. chol. per gramme dried tissue*	*c.p.m. per mg.*	*c.p.m. per mg. chol. per gramme dried tissue*
1	5	20	1,052	27	114
2	5	9	423	22	109
3	5	12	595	28	117
4	2	1	68	1	7
5	2	1	27	4	51
6	5	6	284	3	36
7	5	5	207	2	25
8	24	9	410	8	109
9	24	6	275	9	120
10	2	56	2,498	3	32
11	2	60	2,870	2	28

*Guinea-pigs 1–3 were newborn (less than 1 day old) and weighed 66–80 grammes. Pigs 4–11 were ten days old and weighed 120–150 grammes. Each received $5{\cdot}0 \times 10^6$ c.p.m. $CH^{14}C_2OONa$, pigs 1–9 intraperitoneally, pigs 10 and 11 intracerebrally.

†Calculated from infinitely thick samples of cholesterol digitonide.

in the brain in amounts comparable to that in the liver, indicating that the label penetrated freely into the brain tissue. In keeping with our previous work, even a massive dose of C^{14} acetate injected into an *adult* rat resulted in little incorporation of C^{14} into the lipides investigated (Table III); it

TABLE III.—INCORPORATION OF C^{14} INTO ADULT RAT LIPIDS FOLLOWING INTRAPERITONEAL INJECTION OF A LARGE DOSE OF $C^{14}H_3COONa$*

Organ	*Crude squalene Fraction Total c.p.m.*†	*Crude cholesterol fraction c.p.m. per mg.*‡	*Fatty acid fraction c.p.m. per mg.*
Brain	120	4	15
Liver	163,910	28,534	6,292

*A large female rat received 500 μc/kg. of $C^{14}H_3COONa$ intraperitoneally; sacrificed ten minutes after injection.

†Total counts in non-saponifiable fraction removed from alumina by petroleum ether.

‡Specific activity of non-saponifiable material eluted from alumina with acetone-ethyl ether (1:1), after prior elution with petroleum ether.

seems unlikely at this time that incorporation of any merit occurred in any other brain constituents. It will be noted that the amount of incorporation into both liver and brain cholesterol of the young guinea-pigs (Table II) was considerably lower than that of rats of the same age (Table I), whether the acetate was given intraperitoneally or intracerebrally. Although it seems likely that this difference may be due to the disparity in weights between guinea-pigs and rats at any post-natal age, attention should be called to the work of Schwenk (Schwenk, Alexander and Fish, 1955). These investigators, referring to experiments with the guinea-pig, suggest that "evidently the reactions which convert acetate into cholesterol and this into other products are much slower than in the rat. The guinea-pig does not synthesize cholesterol as easily and it cannot cope with it as well as the rat." A low value of 21 c.p.m. per mg. of cholesterol from the mother guinea-pig liver twenty-four hours after injection of the acetate may substantiate these conclusions.

In both the rat and guinea-pig, sodium acetate injected intraperitoneally into the mother was incorporated into fœtal brain cholesterol (Table IV).

TABLE IV.—INCORPORATION OF C^{14} INTO FOETAL RAT AND GUINEA-PIG CHOLESTEROL FOLLOWING MATERNAL INJECTION OF $C^{14}H_3COONa$*

	Brain		*Liver*	
Rat No.	*c.p.m. per mg.*	*c.p.m. per mg. chol. per gramme dried tissue*	*c.p.m. per mg.*	*c.p.m. per mg. chol. per gramme dried tissue*
1	11	261	6	55
2	10	182	7	40
3	9	169	6	26
4	11	217	6	37
5	6	139	6	48
Guinea-pig No.				
1	9	213	3	11
2	9	232	5	17
3	9	212	3	5

*Rat No. 1 was newly born and was sacrificed when found. All other animals were removed *in utero* and sacrificed immediately. The rats weighed 10–12 grammes, the guinea-pigs 17–20 grammes. After removing aliquots from the non-saponifiable fractions for cholesterol determination, 2·0 mg. of carrier cholesterol was added to the rat and pig brain fractions and 4·0 mg. to the rat liver fractions. No correction is made for these dilutions. Values are reported as free cholesterol calculated from infinitely thick digitonide samples. Maternal rat and guinea-pig each received $5{\cdot}0 \times 10^7$ c.p.m. of $C^{14}H_3COONa$ intraperitoneally.

It will be noted, however, that the specific activity of the fœtal rat and guinea-pig brain cholesterols were not greatly different, and the liver values were not grossly discrepant in contrast to the data obtained with older animals. Whether this is related to the fact that the fœtal weights are approximately the same or to other factors (discussed by Schwenk) cannot

be answered at this time. Popják had previously shown that C^{14}-labelled acetate crossed the placental barrier and was incorporated into cholesterol of various tissues of the fœtus (Popják, 1954); fœtal brain was not investigated. While our results are not entirely surprising, based on the work of Popják, experimental verification seemed needed. In the fœtal rat and guinea-pig as well as the post-natal animals there seems to be little or no blood-brain barrier to sodium acetate.

In both the young rat and guinea-pig (Tables I and II) incorporation of acetate into brain cholesterol was more effective by the intracerebral than the intraperitoneal route. This is presumably due to the more ready availability of substrate to the brain when presented by intracerebral injection. It will be noted that the brain and liver cholesterol specific activities of the ten-day-old rats and guinea-pigs are approximately the same when expressed as c.p.m. per mg. of cholesterol. Thus the acetate is freely available to brain as well as liver in both rats and guinea-pigs of this age. Expressed as c.p.m. per mg. of cholesterol per gramme of tissue, however, the values for brain become proportionately higher than those of the liver in both species of animal, due to the higher concentration of cholesterol in brain than in liver. This factor should be considered in comparing the specific activities of brain and liver cholesterol in tracer experiments, since the total quantity of sterol in the respective organs differs by a factor of about ten. Data for the experiments in which 2-C^{14}-sodium acetate was injected intraperitoneally following intracerebral injection of water are shown on Table V. None of the rat brains examined contained more than a trace of C^{14} in the lipid fractions investigated, an amount corresponding to that found when an equivalent quantity of labelled acetate was given intraperitoneally to normal, unoperated rats. Simple wounding of the brain did not permit acetate from the systemic circulation to enter the brain

TABLE V.—INCORPORATION OF C^{14} INTO ADULT RAT BRAIN LIPIDS FOLLOWING INTRAPERITONEAL INJECTION OF $C^{14}H_3COONa$: EFFECT OF BRAIN WOUNDS*

		Specific Activity, c.p.m. per mg.		
Rat No.	*Time sacrificed (min.) after intraperitoneal injection*	*Crude Non-saponifiable fraction*	*Crude cholesterol fraction*†	*Fatty acid fraction*
1	10	—	1	12
2	10	—	1	4
3	10	10	—	12
4	20	10	—	12
5	60	6	—	12
6	120	10	—	14

*Each rat received 0·1 ml. of distilled H_2O intracerebrally ten minutes prior to the intraperitoneal injection of $1{\cdot}0 \times 10^7$ c.p.m. of the labelled acetate.

†Counted as free cholesterol after conversion from the digitonide via the pyridine method.

through the wound area and become incorporated into the non-saponifiable material of the tissue. Therefore events coincident to the wounding (macrophage infiltration, etc., Macklin and Macklin, 1920) are not responsible for the cholesterol biosynthetic phenomenon.

Although only a few experiments were performed with adult mice, the results (Table VI) in general parallel our findings with adult rats; namely,

TABLE VI. INCORPORATION OF C^{14} INTO MOUSE BRAIN AND LIVER CHOLESTEROL FOLLOWING INTRAPERITONEAL INJECTION OF $C^{14}H_3COONa$*

Mouse No.	Time of Sacrifice (min.) after C^{14} injection	Specific activity of crude cholesterol, c.p.m. per mg.† Brain	Liver
1	5	3	---
2	10	4	26
3	15	4	---
4	20	8	175
5	30	7	211
6	60	9	25

*Each mouse received 1.0×10^7 c.p.m. of $C^{14}H_3COONa$.

†Measured in non-saponifiable fraction eluted from alumina with acetone-ethyl ether (1:1) after prior elution with petroleum ether.

little incorporation of C^{14} labelled acetate into brain cholesterol when injected intraperitoneally as compared to the amount incorporated into liver cholesterol during the same period. Cholesterol-C^{14} values following intracerebral injection of C^{14}-acetate were disappointingly low (unpublished observations). This may reflect the difficulty in performing the injection of fluid properly into the brain area of such a small animal. Some interesting comments by Okita and Kabara (1959) will merit further investigation with this mammal. Other (unpublished) observations include the fact that, as with rat brain tissue, adult mouse, guinea-pig and hamster brain tissue does not incorporate C^{14}-labelled acetate into cholesterol *in vitro*.

DISCUSSION

At the present time there is no satisfactory biochemical explanation for the fact that under most experimental conditions cholesterol in the adult central nervous system appears metabolically inert. All observations on the rat, at least, are in agreement that "turnover" of cholesterol in the adult brain must be limited, if not negligible, and the implications from these observations are that adult brain tissue has lost its ability to metabolize cholesterol. Yet such a conclusion cannot be entirely valid if one considers the recent experiments from our own laboratory which demonstrate active cholesterol biosynthesis from both acetate and mevalonic acid when these precursors are injected into the intact brain. Again, considering only synthetic capacity, it appears more than circumstantial that squalene is present in adult brain, and that the enzyme isopentenyl pyrophosphate

isomerase, so intimately connected with cholesterol biosynthesis, should also be present in adult brain tissue (Agranoff, Eggerer, Henning and Lynen, 1960). The question then arises, is the "block" in cholesterol metabolism of adult brain due to inability of the tissue to further metabolize cholesterol synthesized by the organ rather than inability of the tissue to synthesize the sterol? Opposing this is the fact that one cannot demonstrate cholesterol biosynthesis in the test tube with any number of adult brain tissue preparations. In considering the latter phenomenon it should be borne in mind that brain as well as other tissue frequently does not perform *in vitro* as expected, and that with respect to cholesterol biosynthesis *in vitro*, adult brain tissues and adipose tissue have much in common. The latter tissue also apparently cannot synthesize cholesterol *in vitro* (Feller, 1954).

In conclusion, the one factor which can at present be established as definitely influencing adult brain cholesterol metabolism—in the broadest sense—is the blood-brain barrier. Other than by prohibiting the entrance of cholesterol precursors to the adult brain, the specific influence of this barrier remains unknown. It appears that we have only scratched the surface in our knowledge of cholesterol metabolism in the central nervous system.

SUMMARY

The blood-brain barrier present for certain dyes in immature rats and guinea-pigs was not effective for tracer doses of 2-C^{14}-sodium acetate since intraperitoneally administered acetate became incorporated into brain cholesterol of these animals as well as into liver cholesterol. When administered to a pregnant rat and guinea-pig respectively, the C^{14}-label crossed the placental barrier, as expected, and became incorporated into fœtal brain cholesterol. Even a massive dose of C^{14}-labelled acetate given intraperitoneally to an *adult* rat produced no marked labelling of the brain lipids investigated. From sham intracerebral injection experiments it was concluded that the incorporation of cholesterol precursors into adult brain cholesterol following intracerebral injection of the precursors is not the result of secondary effects of the brain wound *per se*. Factors affecting cholesterol metabolism in the adult brain are discussed.

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THE *DE NOVO* SYNTHESIS OF STEROLS AND CHOLESTEROL FROM ACETATE-1-^{14}C BY SHEEP AND PIG PLACENTAL AND FETAL LIVER SLICES *IN VITRO**

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ABSTRACT

The ability of sheep and pig placental and fetal liver tissue slices to convert acetate-1-^{14}C to sterols and cholesterol was investigated *in vitro*. Radioactive squalene, lanosterol and cholesterol were isolated in small amounts from each of the tissue extracts after incubation with acetate-1-^{14}C. These experiments indicate that the sheep and pig feto-placental endocrine systems have the potential for *de novo* synthesis of cholesterol from acetate, but the significance of this pathway as a source of fetal or placental cholesterol in each of these species has yet to be determined.

INTRODUCTION

A systematic investigation of *de novo* cholesterol biosynthesis by the human placenta and fetus *in vivo* has established that the midgestation human placenta, in contrast to the midgestation human fetus, does not appear to be capable of synthesizing cholesterol from acetate (1, 2). Based on these studies and the work of Van Lenden et al. (3), the balance of evidence suggests that the *de novo* synthesis of sterols and cholesterol by the midgestation human placenta is of little significance. However, human term placental preparations are capable of converting acetate to squalene, lanosterol and cholesterol

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* Contribution No. 443, Animal Research Institute.

in vitro (4-6), which indicates that the placenta may acquire the capacity to synthesize limited amounts of these compounds at a later stage of gestation. Furthermore, the principal source of placental progesterone is probably derived from circulating maternal cholesterol (7-9). Although it has been demonstrated that a number of mammalian placental preparations are capable of converting pregnenolone to progesterone *in vitro* (10, 11), literally nothing is known of either cholesterol or acetate conversion to progesterone by the placenta or fetus of other mammalian species. The present experiments evaluate the ability of sheep and pig placental and fetal liver tissues to carry out the de novo synthesis of cholesterol from acetate-1-^{14}C *in vitro*.

MATERIALS AND METHODS

Materials

Sodium acetate-1-^{14}C (U.S. 61 mCi/mmol) was obtained from Searle/Searle Corporation, Don Mills (Toronto) Ontario, Canada.

All solvents were used without distillation. Crystalline steroid standards were obtained either from Sigma Chemical Co., St. Louis, Mo., U.S.A., or from Steraloids Inc., Keene, N.Y., U.S.A. Purity was assessed by thin layer chromatography and, if necessary, the steroids were crystallized before use.

Tissue preparation, incubation and extraction

Fetal liver and placenta were removed from a pregnant sheep at 127 days gestation and from a pregnant pig at 112 days gestation, after slaughter. Samples of each tissue were cut into approximately 0.5-mm thick slices with a razor blade. Duplicate samples of each tissue slice preparation (approximately 750 mg) were incubated, initially for 2 hr at 37°C in a Dubnoff incubator with air as the gas phase, with 20 μCi acetate-1-^{14}C in 5.2 ml Krebs-Ringer phosphate buffer (pH 7.4) containing 200 mg % glucose, 10 μmole ATP and 10 μmole NAD. After 2 hr the medium was removed by decantation and replaced with fresh medium containing a further 20 μCi acetate-1-^{14}C. Incubation was continued for another 2 hr, then the medium was again decanted and the two media from each incubation combined. The combined incubation media were extracted three times with twice their volume of ethyl acetate. The ethyl acetate was evaporated and the residue partitioned between hexane - 90% methanol. The tissue slices after

incubation were homogenized in 10 ml ethanol-acetone-ether (4:4:1 v/v/v), centrifuged at 2000 x g for 10 min and the supernatants decanted. This procedure was repeated. The combined tissue slice extracts were evaporated almost to dryness and saponified for 1 hr at 60°C in 5 ml of 15% potassium hydroxide in 50% methanol. The saponified mixture was extracted with ether, the ether evaporated and the residue partitioned between hexane - 90% methanol.

Purification and identification of sterols and cholesterol

The hexane extracts from each incubation were combined and evaporated to dryness. The residue was chromatographed on Silica Gel G (Merck) in the system benzene - ethyl acetate (5:1 v/v), as recommended by Avigan et al. (12). This system gives a complete separation of squalene, lanosterol and cholesterol. Zones of radioactivity corresponding in mobility to each of these compounds were eluted from the silica gel with chloroform-methanol (2:1 v/v).

Identification of cholesterol

The cholesterol fractions were diluted with 200 µg reference cholesterol and subjected to digitonin precipitation, as outlined by Butt et al. (13). The digitonin precipitable material was further diluted with 100 mg reference cholesterol, the dibromide formed and free cholesterol regenerated (14). The cholesterol was then recrystallized to constant specific activity.

Identification of squalene

The squalene fractions were diluted with carrier squalene (25-100 mg) and subjected either to bromination (3) or to hexahydrochloride formation (15). The crystals of squalene dodecabromide or squalene hexahydrochloride were subsequently recrystallized to constant specific activity from different solvent systems (Table 2).

Identification of lanosterol

The lanosterol fractions were diluted with 50 mg carrier lanosterol and recrystallized to constant specific activity from different solvent systems (Table 3), initially as the free compound and finally as the acetate derivative.

Localization and measurement of radioactivity

A Panax thin-layer radiochromatogram scanner (Panax Equipment Ltd., Redhill, Surrey, England) was used to locate radioactivity after thin-layer chromatography. Radioactivity was measured in a Beckman Model LS-250 liquid scintillation counter. Samples were counted in 5 ml toluene containing 4 g omnifluor/liter (New England Nuclear, Boston, Mass., U.S.A.) for sufficient time to ensure a counting error no greater than 5%.

RESULTS AND DISCUSSION

The crystallization data for cholesterol, squalene and lanosterol isolated from the hexane fractions after incubation of acetate-1-^{14}C

with sheep and pig placental and fetal liver slices are presented in Tables 1-3. These compounds were isolated in a radiochemically homogeneous form from all the tissues incubated. The percentage conversion of acetate-1-^{14}C to each metabolite after recrystallization to constant specific activity is presented in Table 4. The figures are minimal conversions, because no account was taken of losses incurred during the isolation procedure.

This study shows that sheep and pig placental and fetal liver tissues are capable of synthesizing sterols and cholesterol from acetate-1-^{14}C in vitro. This small but definitive formation of cholesterol from acetate by each of the tissues indicates the potential for de novo synthesis of cholesterol by the sheep and pig feto-placental endocrine system. However, the contribution of this potential source of cholesterol to the overall availability of cholesterol in the feto-placental endocrine system of each of these species has yet to be determined.

ACKNOWLEDGEMENTS

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[a] In accordance with IUPAC-IUB nomenclature for steroids the following systematic names are given to the compounds referred to in this paper.

cholesterol = 5-Cholesten-3β-ol
lanosterol = 8, 24-Lanostadien-3β-ol
squalene = 2,6, 10, 15, 19, 23-hexamethyl-2, 6, 10, 14, 18, 22-tetracosanehexane

TABLE I

CRYSTALLIZATION DATA FOR RADIOACTIVE CHOLESTEROL[a]

ISOLATED FOLLOWING INJECTION OF SHEEP AND PIG FETAL

[illegible] ACETATE-1-^{14}C

(S.A. [illegible] d.p.m./mg)

[illegible]	[illegible]	NUMBER OF CRYSTALLIZATIONS	
		1	2
Sheep	Placenta	[illegible]	[illegible]
		[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]

[illegible]

TABLE [illegible]

[illegible]

[illegible] FETAL

[illegible] ACETATE-1-^{14}C

(S.A. [illegible] d.p.m./mg)

SPECIES	TISSUE	NUMBER OF CRYSTALLIZATIONS			
		1	2	3	4
Sheep	Placenta	[illegible]	183	[illegible]	-
		[illegible]	[illegible]	161	-
	[illegible]	20	20	[illegible]	19
		[illegible]	31	20	[illegible]
Pig	Placenta	148	151	[illegible]	140
		[illegible]	162	[illegible]	[illegible]
	[illegible]	473	443	[illegible]	482
		565	453	472	482

a. The material isolated from the sheep tissues was recrystallized as the dodecahydride derivative. Material crystallized from, 1) Acetone chloroform 2) Toluene methanol chloroform 3) Benzene methanol-chloroform 4) Toluene.

b. The material isolated from the pig tissues was recrystallized as the hexahydrochloride derivative. Material crystallized from ethyl acetate chloroform.

TABLE 3

CRYSTALLIZATION DATA[a] FOR RADIOACTIVE LANOSTEROL[b]

ISOLATED FOLLOWING INCUBATION OF SHEEP AND PIG FETAL

AND PLACENTAL TISSUE WITH SODIUM ACETATE[c] ^{14}C

(S.A. expressed as d.p.m./mg)

SPECIES	TISSUE	NUMBER OF CRYSTALLIZATIONS			
		5	6	7	8
Sheep	Placenta	1078 1520	1021 1184	1083 1193	1043[c] 1166
		4	5	6	7
	F. Liver	89 136	8 118	94 [illegible]	85[c] [illegible]
		6	7	8	9
Pig	Placenta	184 334	16[illegible] 2[illegible]	166 177	[illegible][c] 16[illegible]
		9	10	11	12
	F. Liver	332 394	331 373	[illegible] [illegible]	315[c] [illegible]

a. [illegible]

b. [illegible]

c. [illegible]

[illegible]

[illegible]

[illegible]

[illegible]

SPECIES	TISSUE	% CONVERSION		
		[illegible]	[illegible]	[illegible]
Sheep	Placenta	[illegible]	[illegible]	[illegible]
	F. Liver	0.03	0.0[illegible]	0.0[illegible]3
Pig	Placenta	0.0[illegible]	0.[illegible]	[illegible]
	F. Liver	[illegible]	0.[illegible]	[illegible]

GASTRIC EROSIONS CAUSED BY HOME-BREWED LAGER

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Summary A 22-year-old man was admitted to hospital because of melæna due to acute gastric erosions. In the three weeks before his admission he had drunk several gallons of home-brewed lager. Investigations revealed that this was contaminated with traces of acetaldehyde and acetic acid which had been produced from ethanol by *Acetobacter melanogenus* infection present in the yeast. Experiments were carried out on the effect of "home-brewed" lager and beer on the stomachs of guineapigs; the incidence of erosive gastritis was significantly greater when the animals were given home-brewed lager.

Introduction

THE fermentation of sugars by yeast to produce ethanol has been known since antiquity. Ethanol is the sole product of yeast fermentation under anaerobic conditions. Other fermentative products—e.g., acetaldehyde and acetic acid—will be produced if the yeast is infected by certain other bacteria.

Seven years ago, restrictions governing the home-brewing of beer were removed, and since then home-brewing has become popular. Many types of brewing-kits have become available. Each includes fresh hops, yeast, malt or malt extract, and salts, which are required for fermentation. The main attractions of these kits are the ease of the operation and their low cost.

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The present studies were prompted by the admission to hospital of a patient with melæna who had recently ingested large amounts of home-brewed lager.

Case-report

A 22-year-old electrician was admitted to Stobhill General Hospital in October, 1970, with abdominal pain, nausea and vomiting, and melæna. There was no history of previous dyspeptic symptoms nor of aspirin ingestion. A few weeks previously he had brewed lager at home, using a home-brewing kit which is widely sold. He had made about 5 gallons of this mixture and had drunk most of it himself over a period of 3 weeks.

On examination the patient was not shocked; blood-pressure 120/60 mm. Hg; pulse 105 per minute. Apart from generalised pallor there was no clinical abnormality. His hæmoglobin on admission was 11·8 g. per 100 ml., and his packed-cell volume (P.C.V.) was 35%. Over the subsequent few days his hæmoglobin fell to 9·8 g. per 100 ml. and his P.C.V. to 33%. A barium meal showed no abnormality of the œsophagus, stomach, or duodenum. Fibroscopy five days after admission revealed that the gastric mucal pattern was normal; the mucosa was not injected, but one superficial erosion was seen in the anterior wall of the pyloric canal. The patient had no recurrence of his symptoms.

The clinical diagnosis was erosive gastritis. It was considered that this could have been caused by the ingestion of the home-brewed lager, and studies were carried out to investigate this possibility.

Materials and Methods

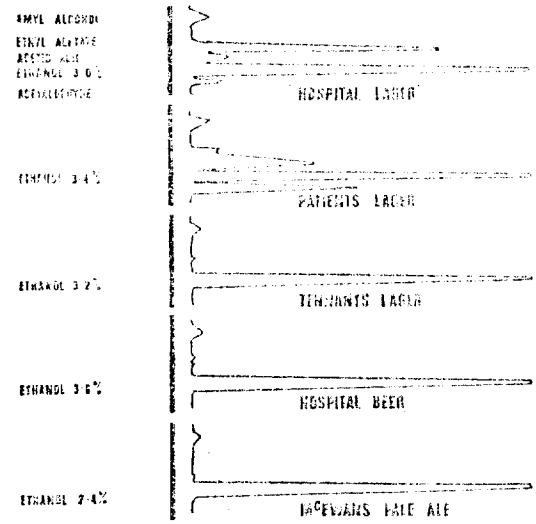
5 gallons of beer and 5 gallons of lager were brewed in the laboratory from a kit similar to the one the patient had used. Samples of laboratory-brewed beer and lager, the patient's lager, and commercial pale ale and lager were analysed by gas-liquid chromatography in a Pye 104 chromatograph by Dr. Hamilton Smith, of the Department of Forensic Medicine, University of Glasgow. The column was 5 ft. long and packed with 'Chromosorb 101'. The resolution temperature was 170°C and the rate of the nitrogen-gas flow was 60 ml. per minute. The internal standard was N-propanol at a concentration of 8 mg. per 100 ml. water.

Samples of the patient's lager, the laboratory-brewed beer and lager, and fresh yeast straight from a kit were analysed bacteriologically by Mr. Peter Owen, of the Department of Microbiology, University of Glasgow.

Animal Studies

36 healthy male guineapigs—weight range 330-580 g.—were fed on the standard guineapig diet 18 based on the recommendations laid down by U.F.A.W.[5] The guineapigs were split into three groups with 12 animals in each group. One group was given measured aliquots of tap-water as their sole drinking-fluid; one group was given measured aliquots of laboratory-brewed lager; and the third group was given measured aliquots of laboratory-brewed beer. In addition, each animal was given a small amount of their appropriate liquid into the stomach. On days 1 and 2 they were each given 2 ml. by intubation tube; on day 3 they were given 4 ml., and on day 4 they were given 5 ml. The animals did not fast before force-feeding but were lightly anæsthetised with ether. The animals were killed on day 5.

In a second experiment, 36 male guineapigs—weight range 250-630 g.—were divided into three groups. The experiment was repeated exactly as before but the animals were not force-fed. The guineapigs were killed by ether anæsthesia and the stomachs were dissected. The gastric mucosa was examined carefully by the naked eye for the



Gas liquid chromatography analysis of beers and lagers.

presence of bleeding-points (defined as the obvious continued oozing of blood from a localised point on the mucosa after careful swabbing). The gastric mucosa of each animal was also tested for the presence of blood by the orthotolidine method.[1] The mucosa was tested by placing drops of the reagent on the mucosa of the whole stomach after removal from the body. In each case the number of seconds taken for the blue colour to develop was estimated by stopwatch.

One stomach from each group of animals in the first experiment—i.e., with intubation—was examined histopathologically. The stomach was not sectioned serially but only random sections were examined. The slides were stained with haematoxylin and eosin.

Results

The results of gas liquid chromatography analysis are shown in the accompanying figure. The peak heights are not proportional to the concentrations of the components in the mixture. The contaminants were present only in trace amounts, and therefore their percentage composition in the lager could not be determined. The ethanol content of all the lager samples was approximately the same. Both the patient's lager and the laboratory-brewed lager had traces of acetaldehyde and acetic acid. The patient's lager had more of these contaminants than the laboratory brew. The laboratory brew also contained traces of ethyl acetate, which could have been formed by enzyme action esterification of the acetic acid. The laboratory-brewed beer was free from contaminating substances. The laboratory-brewed beer was free from bacterial contaminants, but both the patient's lager and the laboratory-brewed lager had an infection of *Acetobacter mellanogenus*, which is known to produce acetic acid from ethanol. The dry lager yeast also had this infection.

TABLE I—NUMBER OF GUINEA-PIGS WITH VISIBLE BLEEDING-POINTS ON GASTRIC MUCOSA (ALL ANIMALS FED DIET 18+CABBAGE)

Drinking-fluid	12 animals/group with intubation	12 animals/group without intubation
Beer	6	5
Lager	7	5
Water	2	1

TABLE II—COMPARISON OF BLEEDING OF GASTRIC MUCOSA BY THE ORTHOTOLIDINE TEST ON GUINEA-PIGS' STOMACHS (ALL ANIMALS FED DIET 18+CABBAGE)

	With intubation			Without intubation		
	Beer	Lager	Water	Beer	Lager	Water
Negative	XX	..	XXXXXX	XXXX	XX	XXXXXX
Positive:						
30 sec.	XXXXXX	XXXXX	XXXX	XXXXX	XXXX	XXXX
25 sec.	XX	XX	X	XX	XXXXX	X
20 sec.	X	XXXX	..	X	..	..
10 sec.	..	..	..	..	..	..
0 sec.	..	..	..	..	..	..

Each cross represents 1 guinea-pig.

On histopathological examination, the sections of the stomachs from the beer and lager drinking animals showed much greater congestion than the stomachs of the water-drinking animals. The congestion was superficial and there was no cellular evidence of infection.

Bleeding-points were found significantly more often in the alcohol-drinking animals which were also receiving aliquots by intubation tube. When the animals were not force-fed there was no significant difference in the number of visible bleeding-points (table I).

TABLE III—RESULTS OF MANN-WHITNEY TESTS

	U value	P
With intubation:		
Lager and water	16	<0·02
Beer and water	35	0·1
Lager and beer	36	N.S.
Without intubation:		
Lager and water	32	<0·05
Beer and water	50	N.S.
Lager and lager	55·5	N.S.

N.S.: not significant.

The results of the orthotolidine tests on the gastric mucosa are shown in table II. The differences between these patterns were analysed by the Mann-Whitney test (table III). The contaminated lager was significantly more irritant than the beer ($P<0·02$) in intubated animals. In the second experiment, when the animals were drinking the fluids but not being force-fed, there was a significant difference between the lager-drinking group and the water-drinking group, but not between the beer and water drinking groups.

Discussion

The results suggest that laboratory-brewed lager is more irritant to the gastric mucosa than laboratory-brewed beer. This difference could be attributed to the harmful contaminants—acetaldehyde and acetic acid—present in the lager. These contaminants were present in trace amounts only, but it is known that acetaldehyde is extremely toxic.

The long-continued use of strong alcoholic beverages in man may result in chronic gastritis in approximately 1 out of 3 individuals.[2] This estimate is valid so long as the alcoholic beverages are not contaminated with other harmful substances. The laboratory-brewed beer was not contaminated and did not produce any

significant irritation on the gastric mucosa of guinea-pigs over a period of four days, in contrast to the contaminated beverages.

Home-brewing of beer, lager, and also wine has become very popular since the introduction of these kits. The main advantage is the low cost, but it now appears that home-brewing is not without danger, particularly in the bacterial contamination of the yeast, which occurred in all three of the samples of lager yeast. If the yeast is contaminated, the liquid will taste and smell sour and this should deter the consumer. These findings suggest that the consumer requires to be protected, either by public-health legislation or by a greater measure of quality control by the producer.

This work was aided by a grant from the Medical Council on Alcoholism.

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RUBELLA VIRURIA IN A 29-YEAR-OLD WOMAN WITH CONGENITAL RUBELLA

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Summary Rubella virus was isolated from the urine of a 29-year-old woman with congenital rubella during an illness in which an upper-respiratory-tract infection was complicated by cardiac arrhythmia, cardiac dilatation, and peripheral oedema. There was no serological evidence of recent rubella infection. It is postulated that intercurrent infection may have led to increased shedding of congenitally acquired rubella virus in this patient's urine.

Introduction

RUBELLA virus persists in the tissues of some congenitally affected patients for months or years after birth. The longest proven postnatal persistence has been 3 years.[1,2] We have suggested that congenitally acquired virus may persist into adulthood, and for this reason we regularly examine virologically the urine of our patients of all ages with congenital rubella.

Case-report

The patient (case 47 in our series [3]) was born (weight [illegible]) on April 11, 1941. There was a history of maternal rubella at 6 weeks' gestation. She had a left cataract, bilateral deafness, and a "slight" cardiac murmur. She progressed well; the cataract was removed and there were no serious illnesses in childhood.

The patient first presented in August, 1957, when she was 26. She had severe bilateral deafness, a moderate speech defect, left aphakia, and right rubella retinopathy. Her blood-pressure was 150/100 mm. Hg and there was a grade-1 systolic ejection murmur parasternally in the 4th intercostal space. Routine tests, chest X-ray, and electrocardiogram (E.C.G.) were normal.

In April, 1968, her blood-pressure was 145/95 mm. Hg and tests were again normal.

On June 1, 1970, she returned for follow-up. She had had an upper-respiratory-tract infection the previous week and had been using ephedrine nasal drops. She complained of ankle swelling but not of breathlessness. There was severe pitting oedema of the feet and legs up to the knees. The jugular venous pressure was elevated 4 cm. and the radial pulse was irregular at 100 beats per minute. Her blood-pressure had risen to 170/100 mm. Hg. No râles were audible. Chest X-ray showed an increase in cardiac diameter and pulmonary vasculature compared with 1957. E.C.G. showed sinus rhythm with frequent atrial ectopics and left-axis deviation, but no other abnormality. Tests, including antistreptolysin-O titre, serum-cholesterol, and serum-proteins, were normal. Urinalysis showed a trace of protein. Microscopic urine examination revealed 3–5 leucocytes and 8–9 epithelial cells per high-power field; there was no bacterial growth on culture but rubella virus was isolated. The patient was treated with bed rest, withdrawal of the ephedrine nasal decongestant, and 2·5 mg. of bendrofluazide daily. A diuresis occurred. By June 22 there had been a weight loss of 2·5 kg.; her blood-pressure had fallen to 155/95 mm. Hg, but the atrial extrasystoles persisted. Chest X-ray showed a reduction in cardiac diameter. Digoxin and potassium supplements were given.

On Aug. 26, after all drugs had been withdrawn, the patient was clinically well and her weight had fallen another 1·5 kg. However, the atrial arrhythmia persisted. T_3-resin uptake and serum-thyroxine were normal. Mantoux test and serological tests for toxoplasmosis and Coxsackie B_1–B_6 were negative.

1 year after the acute episode, although the patient was clinically well, her pulse remained irregular and her blood-pressure was 160/100 mm. Hg. E.C.G. showed atrial and ventricular extrasystoles and left-axis deviation. Chest X-ray, intravenous pyelogram with water load, glucose tolerance test,[4] and serum-creatinine were normal. Urine still contained numerous epithelial cells but was sterile on culture.

Virology and Serology

Rubella virus was isolated [5] from specimens taken to the laboratory immediately after collection.

The urine specimen collected on June 1, 1970, was treated with 0·6% sodium bicarbonate and millipore filtered; 0·3 ml. was inoculated into duplicate tube cultures of the RK_{13} continuous line of rabbit-kidney cells. Within 14 days the cytopathic effect typical of rubella virus was present. The specimen was passaged by freezing and thawing and the cytopathic effect reappeared on the third day. After three passages, the cytopathic effect was shown to be due to rubella virus by neutralization against a standard rubella antiserum prepared in rabbits. Rubella virus was reisolated from a portion of the original specimen which had been kept at −20°C for 16 days.

During the 3 months from June 1, 1970, to Aug. 26, 1970, eight other urine specimens and six postnasal swabs were collected—rubella virus was not isolated from any of these specimens, nor was it isolated from a urine specimen collected in April, 1968.

Rubella haemagglutination-inhibition (H.I.) antibody titres were estimated using a modification [6] of Flotkin's method.[7] When 5 serum samples collected serially in 1970, and one collected in 1967, were tested in parallel, each had a rubella H.I. antibody titre of 1/40. Using a 'Sephadex G-200' column for separation of IgM antibody,[8] no IgM

CLINICAL APPLICATION OF THE BACTERICIDAL EFFECT OF ACETIC ACID IN EXTERNAL OTITIS.

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In a recent issue of this Journal, Jones[1] posed the question as to whether acidity *per se* was a determining factor in controlling ear canal infections. *In vitro* evidence was presented, indicating that it was not the acidity but the acetic acid molecule that effected bacterial control.

Others[illegible] have carried out similar *in vitro* studies showing that acetic acid even in dilute solution kills bacteria, whereas other stronger acids are not effective. The author[illegible] applied this principle of the molecular effect of acetic acid in the treatment of external ear infection. This disease is ideally suited to treatment with acetic acid, because the invading organism is usually a gram-negative bacillus. These organisms are particularly sensitive to the effect of the acetic acid molecule.

Initial work was done with aqueous acetic solutions which were effective but had limitations, such as the necessity of frequent administration, maceration of tissue, and adherence of the cotton wick used when the patient neglected to maintain the moistening procedure. To remedy these disadvantages and to add positive benefits, a mixture of acetic acid in propylene glycol was devised. This can be applied in a more concentrated solution, thus requiring less frequent administration. The propylene glycol has a drying effect on the exuding inflamed skin of the infected ear canal. The glycol acts as a lubricant to assist the insertion of a snugly fitting wick into the ear canal; it prevents adhesion of the wick fibers to the healing skin; further, part of the acetic acid is chemically loosely combined with the propylene glycol to form 1.2 propanediol diacetate ester and this helps reduce

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the otherwise irritating quality of a concentrated acetic solution. The ester readily dissociates into acetic acid and the glycol at the wet skin surface.[illegible]

Owen,[2] working in the Massachusetts General Hospital, showed that in a weak buffering media, as little as 0.3 per cent acetic acid inhibits the growth of all organisms. The complement to this fact is that human skin will grow in 0.5 per cent acetic acid. This was shown by Ludwig[9] who used a 0.5 per cent acetic acid solution to moisten a wet dressing, immobilizing split skin grafts. In 23 cases he found that the skin took and grew in the acetic acid solution.

The author,[illegible] working in the surgical clinic of the Johns Hopkins Hospital, was able to grow a pinch graft on a varicose ulcer, using a 2 per cent acetic acid solution contained in a carboxy methyl cellulose aqueous gel. This work demonstrates the fact that solutions of acetic acid, which inhibit the growth of all organisms, are innocuous to skin; and because acetic acid is a part of body metabolism, the patient can not be allergic to it. To date, with extensive experience from many investigators, not a single case of allergic irritation has been reported. A series of 142 successive ears was treated with the previously described formulation without a single failure.[illegible] Since using this mixture the author has treated 860 cases of acute diffuse external otitis without a single failure to control the infection. Jenkins[11] reported on a series of 200 cases without a failure and other series, as yet unreported, are being developed.

The unusual and dramatic success of this treatment applies only to the acute diffuse external otitis commonly seen in the summer and often referred to as "swimmer's ear." In the treatment of furunculosis this regimen is as effective as other topical methods; but, because the disease involves the deep layers of the skin, surface medicants do not penetrate, and the disease is more effectively treated by systemic antibiotics and surgical drainage.

External otitis is a dermatitis and is best treated with a wet dressing, as are acute eruptions elsewhere on the body.

*Supplied as Vo Sol by Wampole Labs.

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The ear canal has an advantage, in that the wet dressing can be applied as a wick. One simple point needs to be stressed and that is that the solution should be instilled by the physician to insure the complete saturation of the wick. Special effort must be taken to eliminate air pockets in the depth of the canal which prevent the penetration of the solution. Slight negative and positive pressure often has to be applied until the air ceases to bubble up from the distal part of the canal. After the wick is thoroughly saturated it is a simple matter for the patient to maintain saturation by the periodic addition of a small amount of medicant. If this precaution is observed, consistently good results are obtained.

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Activation of Pitressin by Acetic Acid.*

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In the process of development of a technic for the extraction of pitressin from tissues as a preliminary to its assay, the effect of acetic acid on pitressin *in vitro* was studied.

* This work was assisted by the John and Mary Markle Foundation and the Board of Research of the University of California.

TABLE I.

Activation of Pitressin by Acetic Acid.

Preparation (1)	Dose given, M.U. Treated (2)	Dose given, M.U. Control (3)	Dose assayed, M.U. Treated (4)	Dose assayed, M.U. Control (5)	Activation factor Col. 4/Col. 2 (6)	Activation factor Col. 4/Col. 5 (7)
M.U.						
150 boiled c* 50 cc 1% acetic acid	13.95	—	45	—	3.2	—
1000 " c 10 " " "	17.12	10.16	30.5	12.0	1.7	2.5
400 refluxed 15 min c 25 cc 1% acetic acid	4.68	4.70	8.1	11.4	1.7	0.71
400 " " " " " " "	4.52	4.56	9.6	5.4	2.1	1.8
400 " " " " " " "	8.31	8.76	13.2	8.3	1.6	1.6
400 " " " " " " "	8.52	8.20	11.7	7.4	1.4	1.6
4000 " " " " " " "		Pressor Assay			1.6	1.6
4000 " " " " " " "	8.09	7.68	15.3	9.0	1.9	1.7
4000 " " " " " " "		Pressor Assay			1.6	1.5
5000 " 10 " " 25 " " "		"			1.7	1.3
4000 " 15 " " " " "	13.57	13.10	9.0	10.8	0.66	0.83
1000 mixed cold c 10 cc 1% acetic acid	4.01	3.99	1.5	5.8	0.37	0.26
400 refluxed 15 min c water		Pressor Assay			0.5	0.35
400 " " " " 1 N HCl		"			0.8	0.8
400 " " " " 15 N Sod. Acetate		"				

The antidiuretic assays (Barnes) were made on a newly calibrated group of rats and doses of treated pitressin were adjusted with doses of untreated pitressin.

The pressor tests were made by intravenous injection into deeply chloralized dogs of alternate similar doses of treated and untreated pitressin (Hamaoka). The activation factor in these instances was obtained from the ratio of the maximum blood pressure rise obtained with the treated and treated subtracted (Column 7), and checked by a ratio of the areas under the pressure curves (Column 6).

Columns (2) and (3) are in doses per rat. The paired doses are the actual ratio weight basis.

c = with.

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Hamaoka O., *J. Am. Chem. Soc.*, 1928, 50, 573.

Table I shows the result of the assays of a variety of preparations.† Note in every case that the pitressin which had been boiled with acetic acid appears to have a definitely more potent effect than untreated pitressin, but that the other treatments involved a loss of potency. Column 1 indicates that this activation is a result of boiling with acetic acid rather than of boiling, concentration or acetate ions alone. The close similarity of the activation as measured by the antidiuretic method and by the pressor method is to be noted, though this does not necessarily indicate that the two effects (antidiuretic and pressor) are due to the same substance.

The failure of hydrochloric acid to activate pitressin (confirming Dale and Dudley[1] and others) suggests that the mechanism is not a hydrolytic one. The fact that the effect is observed equally with subcutaneous injection in the one assay and with intravenous injection in the other precludes the likelihood of the activation depending upon variations in the rate of absorption.

Noble, Rinderknecht and Williams,[2] observing potentiation of pitressin solutions assayed by the antidiuretic technic after treatment with certain heavy metal acetates, concluded that the effect of these augmenting substances depended on changes in the rate of subcutaneous absorption. The present investigation indicates the possibility that the observed augmentation may have been due to the effect of acetic acid derived from hydrolysis of the heavy metal acetates on the pitressin rather than to the effects of the salts on the rate of subcutaneous absorption.

Since acetic acid, which is commonly employed in the extraction of tissues for assay of pitressin content, is able to potentiate pitressin to a varying degree, there is a possibility that misleading results might be obtained by the application of this method unless extreme care is used in the standardization of all details such as the concentration of reagent and duration of procedures.

Summary. Pitressin when boiled with acetic acid becomes more potent as shown by subcutaneous antidiuretic assay and intravenous pressor assay. These effects are not due to boiling, concentration, acidity, or the presence of acetate ions. The significance of these findings in connection with the preparation of acetic acid extracts of tissues for pitressin assay is discussed.

† These preparations were all made by dilution from pitressin generously given to us by Dr. Oliver Kamm of Parke Davis Laboratories to whom our thanks are extended.

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Differential Healing Periods of the Acetic Acid Ulcer Model in Rats and Cats

It is well known that submucosal injection of several agents, including silver nitrate, formalin, nicotine, and epinephrine can induce gastric ulcers in experimental cats and dogs[1-3]. These experimental lesions grossly and histologically resemble human gastric ulcers, although they heal rapidly in 2-3 weeks. However, one of the authors (S.O.) recently developed a chronic gastric ulcer model in rats which requires the gastric submucosal injection of acetic acid[4], and produces an ulcer which persists for 150 days. Thus, it was of interest to test the acetic acid ulcer procedure in cats, in which the gross anatomy of the stomach is similar to that of man, and compare the healing periods with the rat.

Materials and methods. Gastric ulcers were induced in male Sprague-Dawley rats (230-260 g) under ether anesthesia as described previously by TAKAGI et al.[4], by injection of 10% acetic acid (0.05 ml) into the submucosal layer of the stomach. After surgery the animals were fed normally (Purina Laboratory Chow) and sacrificed at 5, 60, or 200 days by an overdose of ether. Stomachs were removed and fixed according to the technique of BRODIE et al.[5]. Gastric ulcers were measured, and examined histologically (hematoxylin and eosin stain).

Male mongrel cats (2.3-2.7 kg) were subjected to a similar ulcerogenic procedure with the following variations. Cats were anesthetized by Surital (sodium thiamylal; 40 mg/kg, i.p.) and 20% acetic acid (0.5 ml) was injected into the submucosal layer of the gastric antrum on the lesser curvature. 10% acetic acid was not sufficiently ulcerogenic in the cat. The animals were maintained on a normal diet and 2 or 3 cats per period were sacrificed at 7, 14, 21, 28, 42, or 70 days after surgery by an overdose of Surital. After gross examination of the stomachs and measurement of ulcers, the gastric lesions were studied histologically.

Results. Both rats and cats withstood the procedure well, and adverse overt symptoms were not observed at any time during the experimental periods. The results for rats are delineated in Table I, and for cats are shown in Table II.

Gastric ulcer in rats. 5 rats sacrificed on the 5th day after the operation all had sharply defined, deep gastric ulcers (Figure 1a). Histological examination confirmed that the ulcers penetrated the entire gastric wall, and were characterized as confined perforations. By the 60th day the ulcers were markedly reduced in area and depth due to epithelial regeneration and connective tissue proliferation. However, at 200 days all rats had gross gastric ulcers of varying sizes (Table I). The gross appearance of a 200 day ulcer is illustrated in Figure 1b, and the histological character of this lesion is shown in Figure 2.

Gastric ulcer in cats. At 7 days after the operation large and deeply excavated ulcers (4 mm in depth) were observed in the antrum of the stomach on the lesser curvature (Figure 3). The margins of the ulcers were overhanging and the floor was nodular and covered by necrotic debris, food residue and ingested hair. Histologically the lesion penetrated the entire gastric wall and in one of the cats the base of the ulcer consisted of omental fat adherent to the stomach (Figure 4). At 14 days the ulcer in the cat was smaller, but sharply defined (Table II), and granulation tissue in the lesion approached the level of the surface of the mucosa. The defect size was smaller at 21 days, and newly formed capillaries were observed in the granulation tissue. By the 28th day a small mucosal defect with nodular granulation tissue in the floor was found at the center of the area originally ulcerated. At 42 days after surgery the ulcer was completely healed, i.e., regenerated epithelia covered the entire surface of the ulcerated area. After 70 days the lesions remained healed, and there was no evidence of re-ulceration.

Discussion. The present experiments confirm the production of gastric ulcer in rats by submucosal injection

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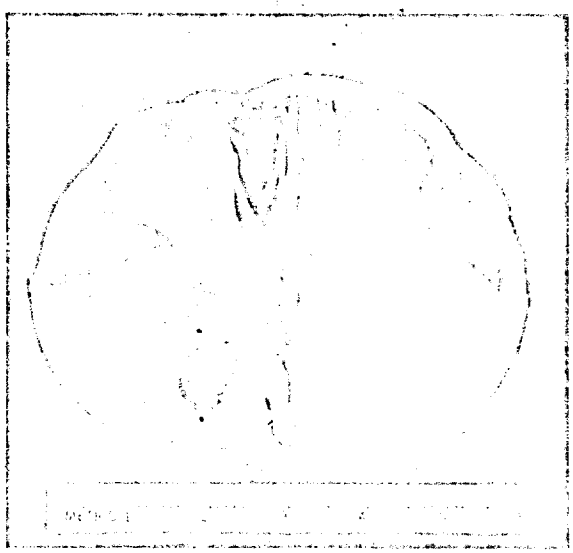

Fig. 1a. Experimental gastric ulcer in the rat at 5 days after induction.

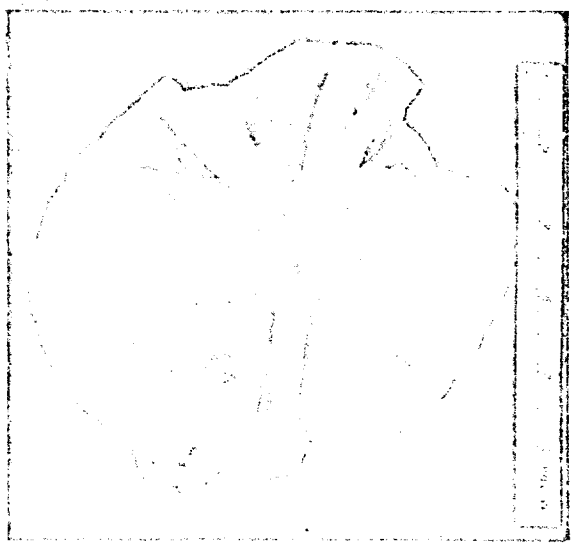

Fig. 1b. Experimental gastric ulcer in the rat at 200 days after induction.

Table I. Healing process of gastric ulcer in rats produced by submucosal injection of 10% acetic acid (0.05 ml)

No. of rats	Days after operation	Diameter of ulcer (mm)	Gross and histologic observations
12	5	10 × 9, 8 × 8, 9 × 9, 8 × 6, 9 × 9, 9 × 9, 9 × 8, 8 × 8, 6 × 5, 6 × 8, 4 × 6, 6 × 4	Punched-out deep ulcer adherent to adjacent liver (confined perforation).
5	60	3 × 3, 1 × 2, 1 × 1, 0, 0	Small, shallow ulcer. Well developed granulation tissue and limited epithelization. Healed ulcer in 2 rats.
4	200	12 × 12, 10 × 10, 2 × 1, 1 × 1	Clearly defined ulcer adherent to liver.

Table II. Healing process of gastric ulcer in cats produced by submucosal injection of 20% acetic acid (0.5 ml)

Cat number	Days after operation	Diameter of ulcer (cm)	Gross and histologic observations
1, 2, 3	7[a]	1.8 × 1.5, 1.9 × 1.1, 1.9 × 1.5	Sharply defined ulcer penetrating the stomach wall, and confined and adhered to liver. Necrotic, nodular floor.
4, 5	14	1.0 × 0.8, 0.9 × 0.7	Marked granulation tissue proliferation at the base.
6, 7	21	0.9 × 0.6, 0.6 × 0.4	Smaller, clearly defined, shallow ulcer with advanced proliferation of mucosal epithelium and connective tissue.
8, 9	28	0.5 × 0.4, 0.2 × 0.2	Small ulcer with necrotic material on surface.
10, 11	35	0, 0	Complete covering of regenerated epithelium on ulcer surface without interposition of muscularis mucosae and muscle layer.
12, 13	70	0, 0	Similar to 35 day response.

[a] Initially, gastric ulcers in cats were of similar size, in proportion to the entire stomach, as rat ulcers.

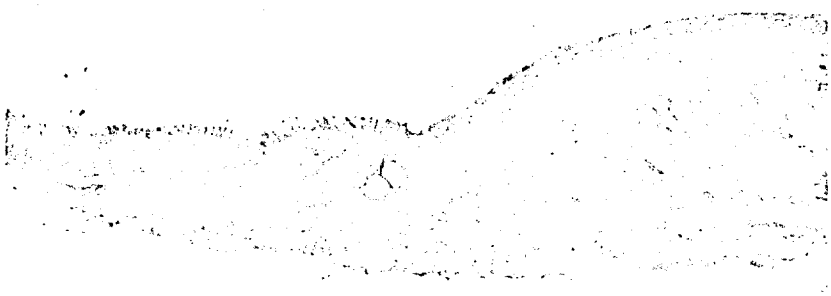

Fig. 2. Photomicrograph of the gastric ulcer in the rat at 200 days after surgery. × 18.

of acetic acid, and demonstrate that these lesions persist for at least 200 days. It seems likely that the experimental ulcer in the rat undergoes partial healing and re-ulceration under chronic conditions since large ulcers were observed at 200 days. In contrast, gastric ulcers produced in the cat were similar in appearance initially, and of similar size in proportion to the stomach, but completely healed at 35 days. The difference in healing was not attributable to the different concentrations of acetic acid used in the rat and cat since a stronger concentration was used with the cat. Although it is possible that the differential healing periods related to the different regions of the stomach in which the ulcer was induced (cat antrum, rat fundus), this is not likely since preliminary studies in our laboratory[6] indicated that acetic acid ulcer healing (painting method[7]) is slower in the rat antrum than in the rat fundus. In the present study the antral region was selected in the cat because human ulcers situated on the antrum are frequently chronic lesions[8]. However, in the rat, which has a much smaller stomach, it is

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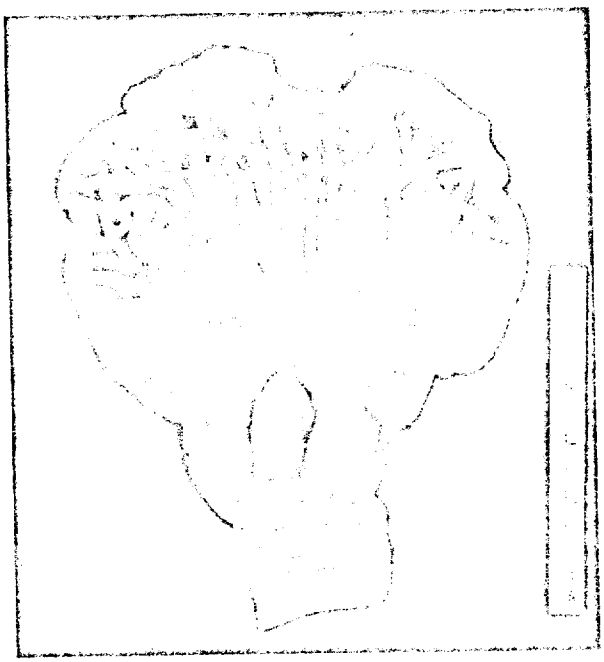

Fig. 3. Gross appearance of gastric ulcer in the cat antrum at 7 days after induction.

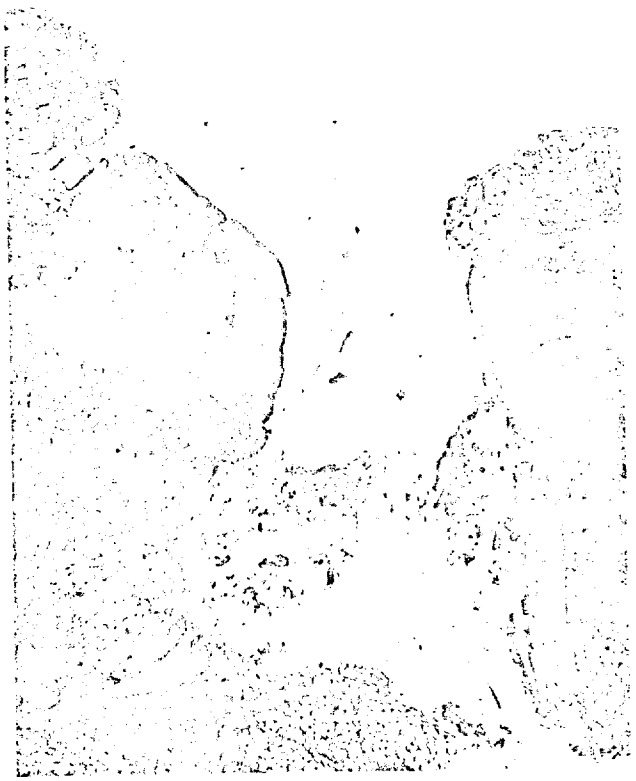

Fig. 4. Photomicrograph of the cat ulcer shown in Figure 3. ×18.

difficult to induce experimental acetic acid ulcers in the antrum by the injection method. The rapid healing of the gastric ulcer produced in cats by acetic acid observed here is similar to the rapid healing reported after nicotine, formalin and silver nitrate injection in cats[4, 5], and after the mechanical induction of gastric defects in cats as described by GUNTER[9]. Thus, it can be concluded that the chronicity of the gastric ulcer in the rat is not necessarily related to the acetic acid itself, but to a special characteristic of the rat. Presumably, normal gastric mucosal regeneration times are similar for both species[10–12]. Inasmuch as the chronicity of the experimental, acetic acid ulcer model in the rat uniquely resembles human peptic ulcer, this model may be quite useful for the study of human ulcer and the evaluation of pharmacologic agents used for this disease.

Zusammenfassung. Methode zur experimentellen Erzeugung eines Magengeschwüres beim Tier, das weitgehend mit dem menschlichen Magengeschwür übereinstimmt.

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Incorporation of Acetate and Fatty Acids into Lipids of Rat Platelets[1] (35377)

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The ability of human platelets to incorporate *in vitro* acetate and fatty acids into their lipids was recently demonstrated by several investigators (1–4). Furthermore, platelets were shown to be the only formed elements of the human blood capable of *de novo* synthesis of fatty acids (5) and were also found to synthesize phospholipids (6). Different fatty acids exhibited distinctive patterns of incorporation into the various lipid classes of human platelets. Considerable variability of these results was observed depending upon the experimental methods used. With the red cells which are only capable of exchange of lipids with the medium, not of *de novo* synthesis, marked species differences were found in their ability to incorporate fatty acids into phosphoglycerides (7). Such comparative studies, however, have not yet been done on platelets. In the present research, using virtually pure platelet suspensions, we have examined the incorporation of acetate and of various fatty acids into different lipid classes of rat platelets and compared the results in these rodents with those reported by other investigators on human platelets.

Materials and Methods. Male Sprague-Dawley rats (280–350 g) were given water and Purina laboratory chow *ad libitum*. After overnight starvation, blood was collected with acid citrate dextrose as anticoagulant; and platelets were separated by differential centrifugation (8). The platelets were washed twice with Gaintner's buffer (9), of pH 6.8, modified to contain no EDTA. Washed platelets from at least four rats were pooled and suspended in modified Gaintner's buffer of pH 7.4 (1 × 10^{9} platelets/ml). Red and white cell contamination was always less than 1 per 25,000 and 1 per 100,000 platelets, respectively. Siliconized glassware was used throughout this procedure.

Palmitic, oleic, linoleic, and linolenic acid-1-^{14}C (Amersham-Searle; Des Plaines, Ill.) and acetate-1-^{14}C (New England Nuclear, Boston, Mass.) were mixed with the respective nonradioactive substance (fatty acids of 99% purity were obtained from Mann Research Laboratories, New York, N.Y.) to obtain the desired specific activity. Fatty acids were bound to 5% bovine serum albumin (BSA, crystallized; Mann Research Laboratories) in 0.1 *M* Tris HCl buffer, pH 7.4. The fatty acids dissolved in benzene were evaporated almost to dryness under a stream of nitrogen, then added to the BSA solution and vigorously agitated on a Vortex mixer for 5 min. Subsequently the remaining benzene was driven off under a stream of nitrogen. In case this solution was not used promptly, it was divided into 0.2-ml aliquots, sealed in ampules under nitrogen and stored in the dark at −20° until used (up to 1 month). During this time, virtually no degradation of the fatty acids in such solutions occurred as shown by gas liquid radio-chromatography (3, 10) (the purity of the fatty acids-1-^{14}C exceeded 95%).

Two ml aliquots of the platelet suspension were incubated at 37° in a metabolic shaking waterbath under an atmosphere of 95% O_2 and 5% CO_2 either with 0.2 ml of aqueous acetate-1-^{14}C, usually 10 μCi, or with 0.2 ml of albumin-bound fatty acid-1-^{14}C, usually [illegible] μCi. Platelets incubated with acetate were washed twice at 4° with 5 ml aliquots of ice-cold modified Gaintner's buffer of pH 6.8; whereas those incubated with fatty acids

[1] Supported by the Atomic Energy Commission Contract AT (30-1)3559

were subjected to five washing with 3 ml aliquots of ice-cold 0.5% BSA dissolved in the same buffer. Following the washing procedure the platelet lipids were extracted by the modified Folch procedure (3) and washed 3 times (11). The extract was evaporated under nitrogen and dissolved in a small volume of extraction solvent. Two small aliquots of this solution were set aside for determination of total radioactivity and PL[2] phosphorus (12) and the remainder was subjected to thin-layer chromatographic separation. Silica gel N-HR (Brinkman Instr., Westbury, N.Y.) was always used as the coating material. The major PL, *i.e.*, SM, PC, PI, PS and PE (Mann Research Laboratories) as well as LPC (Analab, North Haven, Conn.) were used as external reference standards and were resolved by chloroform-methanol-acetic acid-water (75:25:6:2, by vol) (3) (TLC I). The major lipid classes were separated using 2 developing systems according to Skipski *et al.* (18) (TLC II). PL, CEM, and CEB remained at or near the point of application but all the other lipid classes with the exception of CH and DG were clearly separated. With this system monopalmitin, diolein, palmitic acid, tripalmitin, arachidic acid methyl ester, and cholesteryl palmitate (Mann Research Laboratories) were always used as internal reference standards. CH and DG were separated by the method of Freeman and West (14) (TLC III). CEM, CEB, CL, and PA were resolved by the combined use of 2 developing systems: acetone-petroleum ether (1:3, by vol) and chloroform-methanol-acetic acid-water (80:13:8:0.8, by vol) (15) (TLC IV). CEM and CL (Applied Science Laboratories; State College, Pa.) as well as CEB and PA (Mann Research Laboratories) were used as internal reference standards. BHT (4-methyl-2,6-di-tert-butylphenol, Sigma Chemical Co.; St. Louis, Mo.) was added to all developing solvents in a concentration of 50 mg/100 ml.

For determination of radioactivity, the spots on the TLC plates corresponding to the individual lipids were scraped into scintillation vials, suspended in 5% (wt/vol) Cab-O-Sil (Cabot Co.; Boston, Mass) in toluene containing 0.4% (wt/vol) PPO and 0.05% (wt/vol) POPOP and counted in a liquid scintillation spectrometer. Aliquots of the original lipid extract were counted in the above scintillation system without Cab-O-Sil. Quenching characteristics were determined by using an internal standard of toluene-^{14}C.

Phosphorus was determined on each clearly identified major PL and on an area corresponding to LPC (12). Corrections were made for the absorbancy of corresponding areas of blank lanes.

Between 93 and 100% of the radioactivity and of the PL phosphorus spotted onto the TLC plates was recovered after chromatographic separation.

Results. Optimal conditions for incorporation of acetate and fatty acids into platelet lipids were established in preliminary experiments. Acetate and palmitate incorporation into lipids was linear up to 120 min. Changes in pH of the incubation medium from 6.8 to 7.4 did not affect the rate of incorporation of these substances into lipids. Based on these results all subsequent experiments were performed using 2×10^9 platelets suspended in the buffer (pH 7.4) for an incubation time of 60 min.

The rate of acetate incorporation increased linearly with its concentration in the medium up to 0.11 m*M*. At higher concentrations the rate was markedly less but up to 2.2 m*M* no definite saturation plateau was reached. The total incorporation into lipids was 0.1 nmoles/10^9 platelets/60 min at 0.11 m*M* and 0.15 nmoles/10^9 platelets/60 min at 2.2 m*M* acetate in the medium. In order to obtain samples of sufficiently high count rate 0.11 m*M* acetate (sp act. 40 mCi/mmole) was used in all subsequent experiments. The acetate incorporated into lipids constituted about 25% of the total uptake of acetate by

[2] The following abbreviations are used in the text and illustrations. TLC, thin-layer chromatography; FFA, free fatty acids; PL, phospholipids; SM, Sphingomyelins; PC, phosphatidylcholines; PI, phosphatidylinositols; PS, phosphatidylserines; PE, phosphatidylethanolamines; LPC, lysophosphatidylcholines; MG, monoglycerides; DG, diglycerides; TG, triglycerides; CH, cholesterol; CE, cholesteryl esters; FAE, fatty acid methyl esters; CL, cardiolipin; PA, phosphatidic acids; CEM, ceramides; CEB, cerebrosides; PPO, 2,5-diphenyloxazole; POPOP, *p*-bis [2-(5-phenyloxazolyl)]-benzene.

TABLE I. *In Vitro* Incorporation of Acetate-1-^{14}C and Fatty Acids-1-^{14}C into Rat Platelet Lipids and Distribution of Radioactivity Among the Lipid Classes.

	Acetate	Palmitate	Oleate	Linoleate	Linolenate
Total incorporation (nmole/10^{9} platelets)	0.08 ± 0.01[a]	4.2 ± 0.2	2.0 ± 0.2	2.0 ± 0.1	2.0 ± 0.2
		Distribution (%)			
LPC	tr[b]	tr	tr	tr	tr
SM	2.6 ± 0.3	2.5 ± 0.3	1.6 ± 0.1	2.0 ± 0.2	2.0 ± 0.2
PC	20.5 ± 0.6	34.1 ± 0.8	39.8 ± 1.5	49.2 ± 1.0	42.8 ± 1.4
PI + PS	7.0 ± 0.5	1.4 ± 0.1	5.5 ± 0.5	3.7 ± 0.1	3.2 ± 0.6
PE	14.9 ± 0.7	6.7 ± 0.5	11.8 ± 0.7	13.9 ± 0.3	11.4 ± 0.4
CL	tr	tr	tr	tr	tr
PA	tr	tr	tr	tr	tr
CEM	15.4 ± 0.7	2.3 ± 0.1	1.7 ± 0.3	1.8 ± 0.1	6.6 ± 0.5
CEB	tr	tr	tr	tr	tr
MG	5.5 ± 0.1	tr	tr	tr	tr
CH	2.2 ± 0.0	tr	tr	tr	tr
DG	5.0 ± 0.2	5.5 ± 0.5	3.2 ± 0.3	3.1 ± 0.3	10.5 ± 0.8
FFA	1.5 ± 0.5	9.1 ± 1.0	6.5 ± 0.6	6.8 ± 0.3	8.0 ± 0.1
TG	15.6 ± 1.0	36.7 ± 1.0	25.5 ± 1.3	17.1 ± 0.5	9.9 ± 0.7
FAE	tr	tr	tr	tr	tr
CE	tr	tr	tr	tr	tr

[a] Values represent mean ± SEM of 4 expts.
[b] Trace: less than 0.9%.

platelets. With palmitate 0.22 m*M* (sp.act. 10 mCi/mmole) was found to be a saturating concentration, which was then used in all further incubations.

The total incorporation of acetate and various fatty acids into platelet lipids and the distribution of radioactivity among the various lipid classes is shown in Table I. The incorporation of palmitate was highest, 4 nmoles/10^{9} platelets/60 min, about twice that of any of the other fatty acids. *De novo* synthesis of fatty acids by comparison was much lower, only 0.08 nmoles acetate were incorporated into the lipids of 1 × 10^{9} platelets/60 min.

Since the platelet suspensions utilized in these experiments were always contaminated with some red and white cells it appeared of importance to determine the contribution of such blood cells to the observed acetate and fatty acid incorporation into lipids. Rat erythrocytes were found unable to incorporate acetate-^{14}C into red cell lipids. To assess the significance of the contribution of leukocytes to the synthetic activity of platelets the standard system of incubation was modified to contain variable numbers of white cells while holding the platelet concentration constant. There was no significant change in the incorporation of acetate into lipids when white cell contamination was increased up to 7 times its maximal level usually encountered in the platelet suspensions (Table II). Similarly the incorporation of palmitate-1-^{14}C was not significantly affected by red and white cell contamination up to 7 times their usual level (Table II). Based on these findings we feel that neither leukocytes nor red cells in the platelet suspensions contributed significantly to the observed acetate and fatty acid incorporation.

Approximately 40% of the total acetate radioactivity incorporated was found in PL among which PC and PE showed the main uptake (Table I). Among neutral lipids, TG, CEM, and FFA had similar rates of incorporation which were much greater than those of MG and DG. There was clear evidence for CH synthesis although of very limited extent. Each of the fatty acids used showed a distinctive pattern of incorporation into platelet lipids. PC, PE, and TG constituted

TABLE II. Significance of Red and White Cell Contamination of Platelet Suspensions for Acetate and Palmitate Incorporation into Lipids.

Platelets/flask	Leukocytes/flask		Acetate incorporation into lipids (nmoles/60 min)
2×10^9	8.5×10^6		0.15
2×10^9	29.0×10^6		0.14
2×10^9	55.0×10^6		0.15
2×10^9	70.0×10^6		0.15
		Erythrocytes/flask	Palmitate incorporation into lipids (nmoles/60 min)
2×10^9	$<5 \times 10^6$	$<5 \times 10^9$	8.6
2×10^9	18×10^6	18×10^9	8.6
2×10^9	70×10^6	70×10^9	8.8

the principal groups of lipids which utilized fatty acids from the medium and TG exhibited the greatest absolute and relative difference of incorporation among the various fatty acids. The greater the degree of unsaturation of the fatty acids, the lower their utilization in TG. Incorporation either of acetate or of fatty acids into PA, CL, CEB, LPC, FAE, and CE was negligible. The low activity observed by direct counts of these individual spots could also be confirmed indirectly for PA, CL, LPC, and CEB by showing that the difference between radioactivity of the combined major PL classes as separated by TLC I and that of the origin of TLC II containing major PL, LPC, CEM, CEB, CL, and PA corresponded to the radioactivity found in the CEM spot separated by TLC IV.

The specific activities of the major PL classes are shown in Figs. 1 and 2 for platelets incubated with acetate and fatty acids, respectively. When *de novo* synthesis of fatty acids was studied, PI + PS had the highest turnover together with PC ($p<0.2$) and PE ($p<0.1$), followed at a significantly lower level by SM ($p<0.001$). The incorporation of fatty acids into PL showed 50–100 times higher specific activities than were found with acetate.

The various fatty acids displayed certain characteristic differences in their respective specific activities which were most marked when comparing saturated with unsaturated fatty acids. Although all the fatty acids studied had their greatest turnover in PC, palmitate exceeded the unsaturated acids two- to threefold in its turnover rate in SM. It more or less equaled oleate, linoleate, and

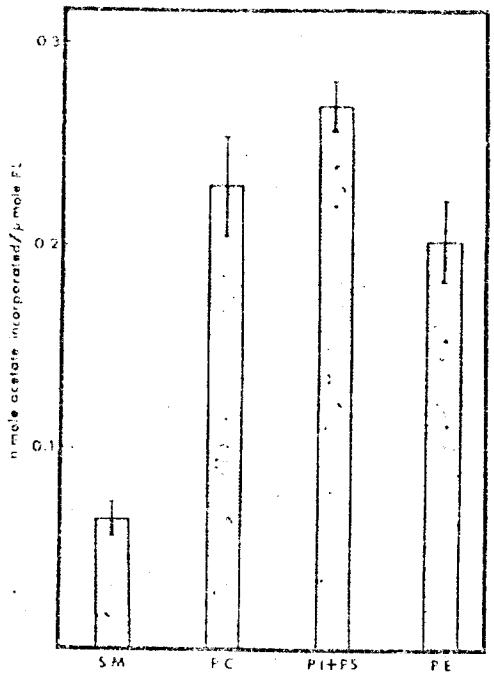

FIG. 1. *In vitro* incorporation of acetate 1-^{14}C into major PL of rat platelets. Two billion washed rat platelets were suspended in 2.6 ml of buffer (pH 7.4) and incubated with 0.25 μmole of Na acetate-1-^{14}C at 37° for 1 hr under 95% O_2 and 5% CO_2. Each bar represents the mean ± SEM of 4 expts.

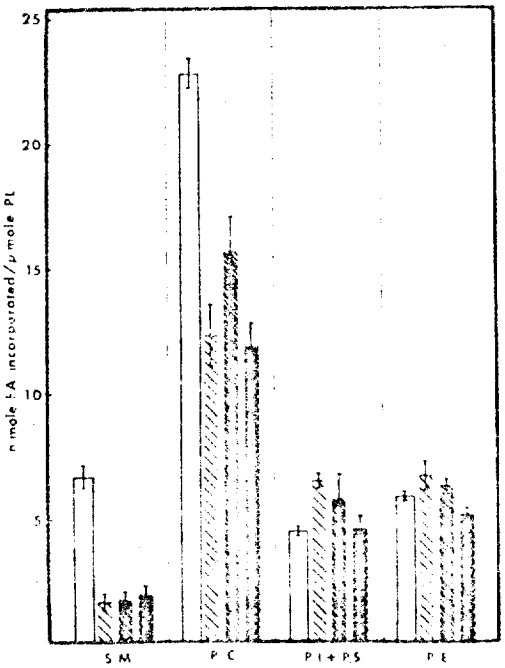

FIG. 1. *In vitro* incorporation of palmitate-1-^{14}C, oleate-1-^{14}C, linoleate-1-^{14}C and linolenate-1-^{14}C into major PL of rat platelets. A mixture of FFA-^{14}C bound to BSA was used in the incubation mixture. Other experimental conditions were identical to those described in legend to Fig. 1. Each bar represents the mean ± SEM of 3 experiments. Open bar: palmitate; single stripe: oleate; double stripe: linoleate; dotted bar: linolenate.

linolenate in PI + PS and PE. Significant differences among the unsaturated fatty acids were found in PC in which linoleate had a higher specific activity than either oleate ($p < 0.05$) or linolenate ($p < 0.02$) and in PI + PS in which oleate was highest being significantly different from palmitate ($p < 0.02$) and from linolenate ($p < 0.02$).

Discussion. These experiments demonstrate that rat platelets are capable of *de novo* synthesis of fatty acids and that certain lipid constitutents of rat platelets are renewed by incorporation of nonesterified fatty acids from the surrounding medium. The maximum rate of acetate incorporation into human platelet lipids was reported to be 0.6 to 0.8 nmoles/10^{10} platelets/hr (1, 3) and thus exceeds by far the maximal synthetic rate in rat platelets which we found to be 0.15 nmoles/10^{10} platelets/hr. A species difference was also found in the relative distribution of acetate among the various lipid classes. While human platelets showed the highest incorporation of acetate into CER, followed by PC and FFA (3), in rat platelets PC was found to have the greatest rate of synthesis followed by TG and CER. Rat platelets were able to synthesize CH similarly to human platelets (3). The distribution of palmitate among the different lipids showed also a striking difference when comparing human (3) with rat platelets. In these, the relative incorporations into TG and FFA were greater and those into PC, PI + PS and CER were smaller than in human platelets. Since the concentrations of TG and DG in rat platelets are much lower than those of PL,[a] it is very probable that the specific activities of the neutral lipids with respect to fatty acid incorporation, particularly of TG, are much higher than those of PL. Substantial amounts of fatty acids were found incorporated into DG which is probable evidence for a high turnover of acyl residues in neutral lipids in rat platelets.

Lipids of rat platelets appear to be in a dynamic state, capable of considerable reacylation by incorporation of fatty acids from the medium and exhibiting a limited ability of *de novo* synthesis of fatty acids. Compared with data from human platelets (3), differences existed in the rate of *de novo* synthesis of fatty acids and in their incorporation characteristics with respect to lipid classes and type of fatty acids utilized.

Summary. The incorporation of acetate and fatty acids into rat platelet lipids was studied by incubating washed platelets with acetate-1-^{14}C or albumin-bound fatty acids-1-^{14}C (palmitic, oleic, linoleic, and linolenic acid) in an artificial medium without addi-

[a] When lipids extracted from 0.4×10^{10} platelets were applied to TLC II, spots of PL and CH were clearly visible after spraying with Rhodamine 6G. However, no other neutral lipids were detected without their internal references. This indicated that concentrations of PL and CH are much higher than other neutral lipids, although no quantitative analysis was performed.

tion of cofactors. Acetate was incorporated primarily into PC, TG, CEM, FFA, and PE which accounted for three-fourths of its total incorporation into lipids. Palmitate incorporation into platelet lipids was twice as high as that of each of the unsaturated fatty acids. A distinctive pattern of distribution of palmitate or of the unsaturated fatty acids among the various lipid classes was observed as well as differences in the relative abundance of the fatty acids incorporated into each PL group. Rat platelets are therefore capable of (i) incorporation of fatty acids from the suspending medium, and (ii) *de novo* synthesis of fatty acids. Pattern of fatty acid incorporation and rate of *de novo* synthesis appear to be different from those in human platelets.

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THE BIOSYNTHESIS OF COENZYME Q IN THE RAT*

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THE contributions of Morton and co-workers at Liverpool (Cain and Morton, 1955; Festenstein *et al.*, 1955) and Crane and co-workers at Wisconsin (1957) to the identification and isolation of coenzyme Q_{10}, the principal mammalian form of the coenzyme, have been presented at this symposium by the investigators. The proof of its structure has been presented by Folkers and co-workers (Wolf *et al.*, 1958) and Isler and co-workers (Morton *et al.*, 1958). Lester and co-workers have also described a series of lower homologues of coenzyme Q, i.e. Q_6, Q_7, Q_8 and Q_9, isolated from a variety of microbiological sources (Lester, Crane and Hatefi, 1958; Lester *et al.*, 1959).

The closeness of the structures of the coenzyme Q family to those of vitamins E and K suggested that coenzyme Q might be a vitamin for the mammal. The studies of Morton and co-workers (1957) with a variety of fat-deficient diets indicated that no dietary source of coenzyme Q was needed by the rat. This raised the question of whether or not coenzyme Q could be synthesized in the tissues of the rat or, like vitamin K, manufactured by its intestinal flora. At the time we became interested in this problem we were studying the relationship of α-tocopherol to other factors which are able to protect the weanling rat from hepatic necrosis on diets low in sulphur amino acids and selenium (Olson and Dinning, 1954; Yang, Riegl and Olson, 1958). We

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discovered that coenzyme Q_{10} could not substitute for α-tocopherol in providing protection against hepatic necrosis in vitamin E-deficient weanling rats, as shown in Table I. Furthermore, as shown in Table II, diets deficient in α-tocopherol did not in-

Table I

PROTECTION OF VITAMIN E-DEFICIENT RATS* AGAINST HEPATIC NECROSIS BY α-TOCOPHEROL AND COENZYME Q_{10}

Supplement	*Dose mg./week*	*Number of animals*	*Percentage survival*
None	0	49	4
α-Tocopherol	2·0	28	100
Coenzyme Q_{10}	2·0	26	12

* Assay animals were vitamin E-depleted weanlings born of mothers given marginal amounts of vitamin E. Necrogenic diet contained soya protein and stripped lard (Olson and Dinning, 1954) and the assay period was 30 days. Supplements were given twice weekly by means of a dropper.

Table II

EFFECT OF VITAMIN E DEFICIENCY AND PROTECTIVE SUPPLEMENTS UPON COENZYME Q CONTENT OF RAT LIVER

(Dose supplement = 6 mg./week)

Supplement	*Number of rats*	*Time in days*	*α-Tocopherol** *μg./g.*	*Coenzyme Q* *μg./g.*
None	12	7	6·1	122
α-Tocopherol	12	14	23·5	118
Coenzyme Q_{10}	6	7	6·7	150
Coenzyme Q_{10}	6	14	6·6	264

* Measured by the method of Swick and Baumann (1952).

fluence the concentration of coenzyme Q in the liver. In addition, added coenzyme Q_{10} had no effect upon the depressed hepatic α-tocopherol levels found in rats fed the necrogenic diet, despite the fact that the exogenous supply of coenzyme Q raised the coenzyme Q levels of the liver. This study supported the view that dietary sources of coenzyme Q were not essential for the rat and that there was no demonstrable interconversion of coenzyme Q and vitamin E in the rat.

Consequently we undertook experiments to study the biosynthesis of this coenzyme in the rat under conditions which minimized a possible contribution from intestinal micro-organisms. The hypothesis which we sought to test is summarized in Fig. 1. Because aromatic biosynthesis (except for partial aromatization of the steroid nucleus in the oestrogens) is unknown in the mammal, it seemed reasonable to expect that some aro-

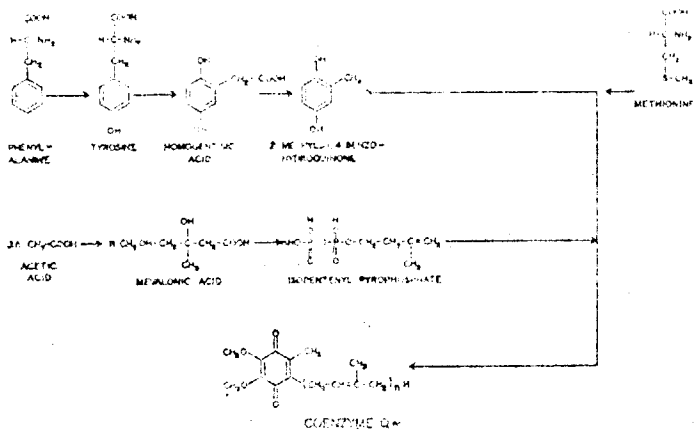

FIG. 1. Hypothetical schema for the biosynthesis of coenzymes Q.

matic compound present in the diet would yield the quinone moiety. Phenylalanine appeared to be an attractive possibility because of its known pathway to homogentisic acid and possibly to a methylbenzoquinone. By analogy with carotenoid and sterol synthesis, it seemed likely that the side chain of coenzyme Q was synthesized from acetate *via* mevalonic acid and a polyisoprenoid pyrophosphate, followed by condensation with the ring compound. The known pathways of O-methylation *via* S-adenosyl methionine (Axelrod and Tomchick, 1958) suggested, finally, that methionine could be the donor of the methyl groups.

In the first group of experiments which we undertook,

[1-^{14}C]acetate and [U-^{14}C]phenylalanine in doses of 50 μc/50 g. body weight were injected intraperitoneally into separate groups of young male albino rats which had had access to food during the preceding night. The rats were killed in 90 minutes, the livers pooled, homogenized, extracted with Bloor's reagent (alcohol : ethyl ether, 3 : 1), and the lipid extract was saponified

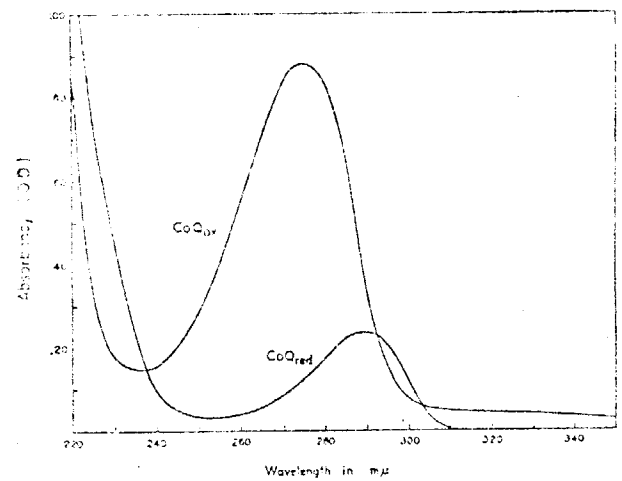

FIG. 2. Absorption spectrum in ethanol of coenzyme Q fraction isolated from rat liver after the fifth alumina column. The spectrum of the reduced coenzyme was obtained after addition of potassium borohydride.

with methanolic potassium hydroxide in the presence of pyrogallol for a period of 1–2 hours at 80°. The non-saponifiable fraction was extracted with petroleum ether and the petroleum ether extract was washed, dried and subjected to chromatography on alumina (Brockman's grade III) (Heaton, Lowe and Morton, 1957) with progressively increasing concentrations of diethyl ether in petroleum ether. The yellow coenzyme Q band was eluted with 4 per cent ether : petroleum ether and was determined spectroscopically using $\Delta E^{1\%}_{1cm.}\,\lambda^{ethanol}_{O\rightarrow R}$ 275 mμ with

potassium borohydride as the reducing agent. Vitamin A and cholesterol were identified in subsequent fractions by the Carr-Price and Liebermann-Burchard reactions.

Cholesterol was purified *via* the digitonide, and the fractions containing coenzyme Q and vitamin A rechromatographed

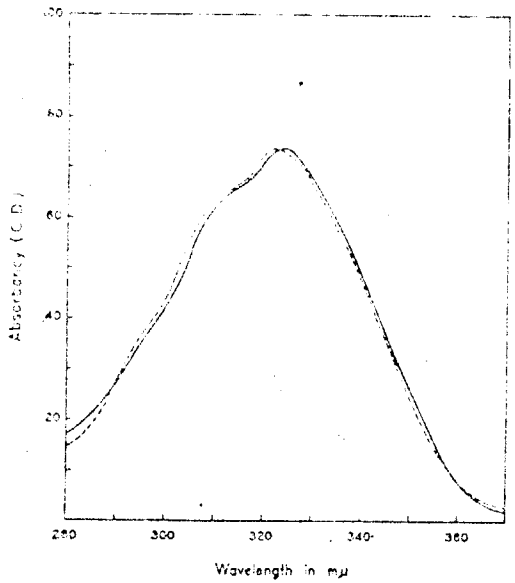

FIG. 3. Absorption spectrum of vitamin A isolated from rat liver (solid line). The spectrum of an authentic specimen of vitamin A alcohol is shown for comparison (dashed line).

to spectral purity. Fig. 2 shows the spectrum of coenzyme Q after the fifth alumina column which had a $\lambda^{\text{ethanol}}_{\text{max.}}$ 275 mμ ($E^{1\%}_{1\text{cm.}} = 183$) and a $\lambda^{\text{ethanol}}_{O \to R}$ 275 mμ ($\Delta E^{1\%}_{1\text{cm.}} = 157$) which were considerably higher than that reported for coenzyme Q_{10} (Crane *et al.*, 1959) and gave us the first hint that lower homologues of coenzyme Q were present in rat liver. Fig. 3 shows the spectrum of vitamin A isolated from normal rat liver in comparison with

the spectrum of authentic vitamin A alcohol. In the early experiments the coenzyme Q fraction from animals given [1-^{14}C]acetate and [U-^{14}C]phenylalanine was simply chromatographed to a constant specific activity on alumina as determined by c.p.m./

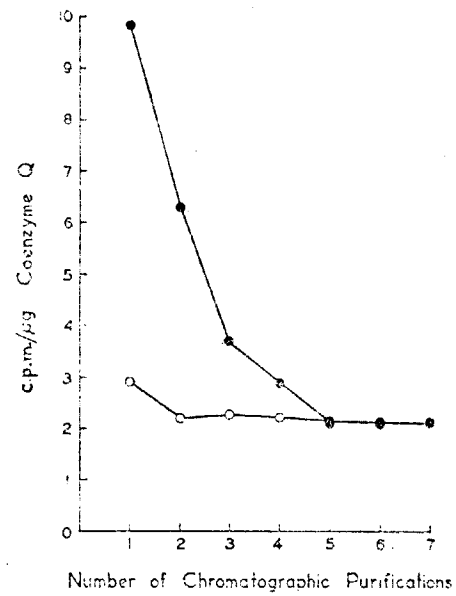

FIG. 4. Effect of repeated chromatography of ^{14}C-labelled coenzyme Q from rat liver on alumina (Brockman's grade III). The solid circles are for the coenzyme Q labelled with [1-^{14}C]acetate. The open circles are for the coenzyme Q labelled with [U-^{14}C]phenylalanine.

$\Delta E^{1\%}_{1\text{cm.}}$ $\lambda^{\text{ethanol}}_{O \to R}$ 275 mμ. All fractions were counted by the liquid scintillation method. The number of chromatographic steps required to reach constant specific activity when these two substrates were used as precursors for coenzyme Q biosynthesis is shown in Fig. 4. It may be seen that the [1-^{14}C]acetate-labelled coenzyme Q had considerably more impurities in the initial

fractions than did that labelled with [U-^{14}C]phenylalanine. Fatty acids were also isolated from the saponifiable fraction. The specific activities of the coenzyme Q, cholesterol, vitamin A and fatty acids when synthesized from [1-^{14}C]acetate and [U-^{14}C]phenyl-

Table III

INCORPORATION OF [1-^{14}C]ACETATE AND [U-^{14}C]-L-PHENYLALANINE INTO COENZYME Q, CHOLESTEROL, FATTY ACIDS AND VITAMIN A

	Coenzyme Q		*Cholesterol*		*Fatty acids*		*Vitamin A*	
	c.p.m./mg.	*μg./g. liver*	*c.p.m./mg.*	*mg./g. liver*	*c.p.m./mg.*	*mg./g. liver*	*c.p.m./mg.*	*μg./g. liver*
[1-^{14}C]Acetate	2,140	22·4	4,841	2·01	1,863	15·3	<20	23
[U-^{14}C]-L-Phenylalanine	2,150	29·4	533	2·25	28	24·9	<20	20

alanine are compared in Table III (Dhalamch and Olson, 1959). It may be seen that the coenzyme Q synthesized from [1-^{14}C]acetate is less radioactive than the cholesterol synthesized simultaneously, whereas the coenzyme Q synthesized from phenylalanine

Table IV

COMPARATIVE RATES OF INCORPORATION OF RADIOACTIVITY FROM [U-^{14}C]-L-PHENYLALANINE AND [1-^{14}C]ACETATE INTO CHOLESTEROL AND COENZYME Q BY RAT LIVER (50 μc./150-g. RAT)

		Radioactivity in fractions		
Number of experiments	*Substrate*	*Coenzyme Q c.p.m./mg.*	*Cholesterol c.p.m./mg.*	*Ratio coenzyme Q/cholesterol*
9	[U-^{14}C]-L-phenylalanine	914 ± 530	350 ± 50	2·64 ± 0·50
6	[1-^{14}C]acetate	3,250 ± 770	2,810 ± 910	1·06 ± 0·34 0·05 > *P* > 0·01

is several times as radioactive as the cholesterol simultaneously synthesized. The ratio of specific activities of the fatty acids paralleled those of cholesterol, and the vitamin A was non-radioactive in both cases. A series of experiments with the two substrates is summarized in Table IV. If one can accept the

cholesterol radioactivity as signatory of the rate of polyisoprene synthesis, these data would suggest that although some of the uniformly labelled phenylalanine was catabolized to acetoacetate and its carbon atoms reincorporated into the isoprene side chain, some direct incorporation of phenylalanine into coenzyme Q had occurred. This finding was then in accord with the hypothesis presented.

Gloor and Wiss (1959*a*) reported that [2-^{14}C]mevalonic acid injected into normal and vitamin A-deficient rats for four days yielded a radioactive coenzyme Q which was isolated from liver, chromatographed on alumina, and crystallized to constant specific activity with carrier Q_{10}. In our own early experiments we frequently added small amounts of coenzyme Q_{10} to aid in the chromatographic purification and this, of course, tended to obscure the anomalously high extinction coefficients obtained with native coenzyme Q from rat liver. The subsequent appearance of the paper by Linn and co-workers (1959) reporting the presence of "small amounts of Q_9" in rat heart focused our attention again on the necessity of positively identifying the coenzyme Q homologues in rat liver.

We consequently undertook a re-examination of the problem with additional chromatographic methods (Lester *et al.*, 1959; see also Linn *et al.*, 1959). The reverse phase paper system we have used most extensively is actually a hybrid of these two methods, employing Vaseline as the stationary phase and *n*-propanol : water, 4 : 1 as the mobile phase. With the aid of this system it was quickly discovered that coenzymes Q_9 and Q_{10} moved together on alumina and could not be separated by repeated chromatography on this adsorbent. The coenzyme Q fraction from rat liver chromatographed to spectral purity on alumina—$\lambda_{O \to R}^{ethanol}$ 275 mμ ($\Delta E^{1\%}_{1cm.}$ = 157)—was then studied by paper chromatography as shown in Fig. 5. The spots were developed with 1 per cent permanganate. The coenzyme Q_9 spot accounted for over 80 per cent of the total as measured by elution and

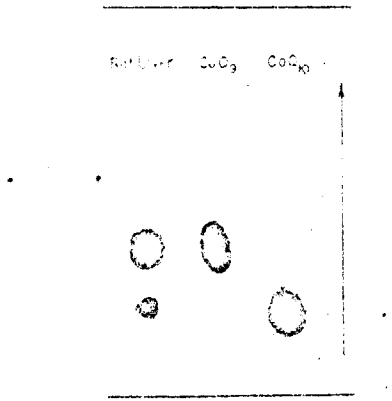

FIG. 5. Chromatography of coenzyme Q fraction from rat liver on paper after purification to spectral purity on alumina. Vaseline is the stationary phase and *n*-propanol : water, 4:1 is the mobile phase. Coenzyme Q_9 and Q_{10} markers are shown.

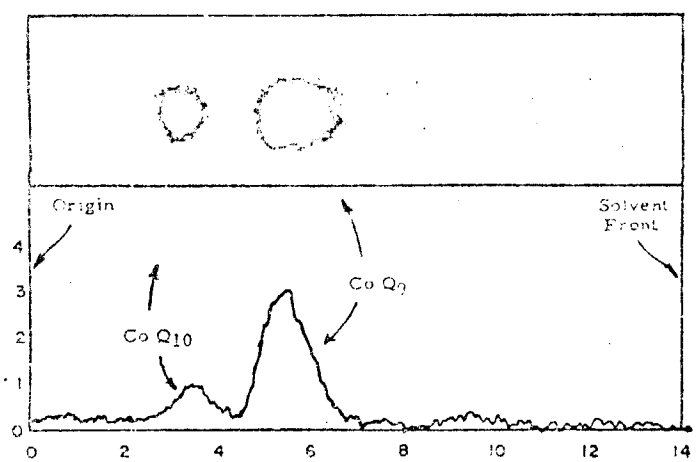

FIG. 6. Densitometric estimation of coenzymes Q_9 and Q_{10} from rat liver on a paper chromatogram. Spots were developed with 2 per cent permanganate.

spectrophotometric estimation or by direct scanning of the paper strip with a Spinco Analytrol as shown in Fig. 6.

Since coenzyme Q_9 had not so far been isolated from normal liver, it appeared important to attempt this. Two kg. of rat liver was extracted, saponified with pyrogallol, and the non-saponifiable fraction was again chromatographed 5 times on alumina; 70 mg. of crude coenzyme Q was obtained with $\lambda_{O\to R}^{ethanol}$ 275 mμ ($\Delta E_{1cm.}^{1\%}$ = 156). This product was then rechromato-

Table V

PROPERTIES OF COENZYME Q_9 ISOLATED FROM RAT LIVER

Compound	*Coenzyme Q_{10} (beef)*	*Coenzyme Q_9 (torula)*	*Coenzyme Q_9 (rat)*
Formula	$C_{59}H_{90}O_4$	$C_{54}H_{82}O_4$	
Carbon per cent	82·13	81·60	81·43
Hydrogen per cent	10·44	10·32	10·24
Methoxyl per cent	7·20	7·8	8·32
M.p.	49°	45°	44–45°
R_F*	0·27	0·36	0·38
$E_{1cm.}^{1\%}$ $\lambda_{max.}^{ethanol}$ (275 mμ)	165	185	188
$E_{1cm.}^{1\%}$ $\lambda_{min.}^{ethanol}$ (236 mμ)	28·4	33·1	30·0
$\Delta E_{1cm.}^{1\%}$ O→R (275 mμ)	142	158	160

* Lester and Ramasarma (1959).

graphed on polyethylene powder using the reverse phase system of Wiss and Gloor (1958), and 40 mg. of coenzyme Q_9 was obtained and recrystallized twice from methanol. These crystals showed only one spot on paper (R_F = 0·38) and had the properties shown in Table V. The infrared spectrum showed the typical bands for coenzyme Q and the amount of absorption at 2,900 cm.$^{-1}$ (the CH_2 stretching frequency) showed a degree of absorption proportional to a compound with a side chain of 9 isoprene units* (Noll *et al.*, 1960).

The finding of coenzyme Q_9 as the principal form of the

* We are indebted to Dr. Noll for these measurements.

coenzyme in rat liver, a discovery also made by Gloor and Wiss (1950a) first in the vitamin A-deficient rat and subsequently (1900) in the normal rat, raised the question of the origin of both coenzymes Q_9 and Q_{10} in this species. Previous evidence suggested that neither was required in the diet. A group of weanling rats was fed chow and Q-free diets (Page *et al.*, 1959) for periods of two months. As may be seen from Table VI, a Q-free diet composed of casein, glucose, lard, salts and vitamins (Olson, Jablonski and Taylor, 1958) did not depress the level of coenzyme

Table VI

CONCENTRATION OF COENZYME Q IN RAT LIVER UNDER VARIOUS DIETARY CONDITIONS

Experiment No.	*Number of rats*	*Diet*	*Daily supplement*	*Coenzyme Q µg./g.*	*Coenzyme Q_9 per cent*	*Coenzyme Q_{10} per cent*
1	6	Chow	—	110 ± 5	82	18
2	6	Q-free*	—	117 ± 8	81	19†
3	6	Q-free*	2 mg. CoQ_{10}	192 ± 4	71	29

* CoQ-free diet was a standard synthetic diet containing 18 per cent casein and 6 per cent lard.

† Faeces from rats on a CoQ-free diet contained 23 ± 1 µg./g., of which 55 per cent was CoQ_9 and 45 per cent was CoQ_{10}.

Q in rat liver nor alter the ratio of the two homologues. The fact that both homologues are present under conditions of Q-free dietary feeding suggests that these compounds are synthesized either by the animals' tissues or by the microbiological flora in the intestinal tract. The faeces obtained from rats fed the coenzyme Q-free diet contained 23 µg./g. of coenzyme Q, of which 55 per cent was Q_9 and 45 per cent was Q_{10}. It would appear, therefore, since both homologues appeared in the faeces, that both could be derived from microbiological synthesis although this did not exclude a contribution from the tissues. When coenzyme Q_{10} was given in the diet in amounts of 2 mg./day for one week, the coenzyme Q content of the liver rose, as indicated previously, and the proportion of the two homologues

changed, but not as drastically as might be expected from the rather large increase in total liver coenzyme Q. It is apparent that homeostatic mechanisms operate in the rat liver to maintain a preponderance of coenzyme Q_9.

It was next decided to repeat the isotopic experiments de-

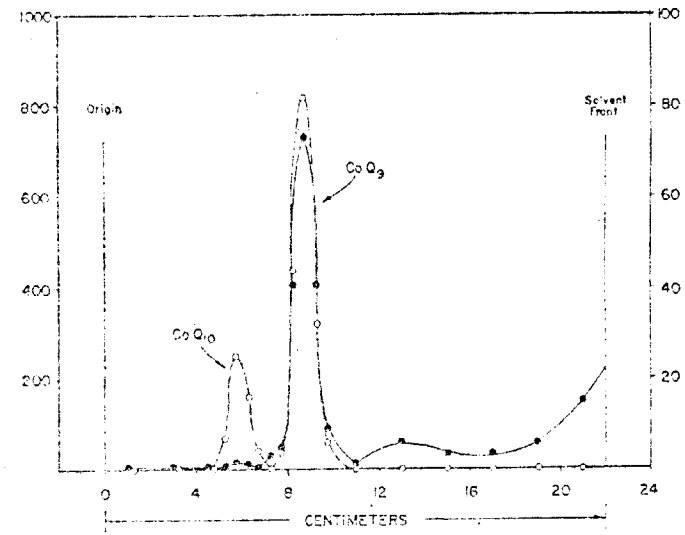

FIG. 7. Radiochromatogram of coenzyme Q fraction of enterectomized rat given intravenous [1-^{14}C]acetate, after purification on alumina. Vaseline-impregnated paper was used and the chromatogram was developed with *n*-propanol : water, 4:1. The solid line represents radioactivity in scintillation c.p.m., the dashed line represents concentration of coenzyme Q (µg.) determined spectrophotometrically.

scribed earlier with [1-^{14}C]acetate and [U-^{14}C]phenylalanine in such a way as (1) to exclude the intestinal tract of the animal, and (2) to monitor the radioactivity in both homologues. An operation was devised to remove the intestinal tract from the rat from the duodenum to the rectum and, at the same time, preserve the arterial blood supply to the liver. The portal vein, superior and inferior mesenteric arteries, and splenic artery and

vein were ligated, and the spleen and complete large and small intestine removed under Nembutal anaesthesia (40 mg./kg.). The isotopic substrate (50–500 μc/50 μM) was given intravenously to young adult rats averaging 150 g. in weight, and the animals were killed in 90 minutes. After chromatography to constant specific activity on alumina, the coenzyme Q fraction was chromatographed on paper, using Vaseline and *n*-propanol : water, 4 : 1. The distribution of radioactivity and coenzymes Q_9 and Q_{10} on this chromatogram, with [1-^{14}C]acetate as substrate, is shown in Fig. 7. The radioactivity was primarily in Q_9, although there were some unidentified fractions on the chromatogram which moved ahead of Q_9 but which had no typical absorption bands for coenzyme Q and probably represent metabolites or precursors of the coenzyme of similar polarity. Table VII shows the results of three experiments with radioactive acetate at different levels of isotopic dosage in which the labelling of cholesterol is compared with that of Q_9 and Q_{10}. The first experiment was in a normal animal and the second two were done in enterectomized animals. Cholesterol was very well labelled in these experiments and was approximately 2–3 times as active as coenzyme Q_9. Coenzyme Q_{10} was relatively cold, approximately one-thirtieth the activity of coenzyme Q_9. Whether this low activity represents real *in vivo* biosynthesis is yet to be established. Since the size of the liver plasma pool of

Table VII

INCORPORATION OF RADIOACTIVITY FROM [1-^{14}C]ACETATE INTO HEPATIC CHOLESTEROL AND COENZYMES Q

Experiment No.	Radioactivity administered as [1-^{14}C]acetate μc	Radioactivity of isolated products: Cholesterol c.p.m./mg.	Coenzyme Q_9 c.p.m./mg.	Coenzyme Q_{10} c.p.m./mg.
1	50	1,460	750	30
2	500	37,100	11,600	330
3	500	25,000	9,800	270

cholesterol is about 20 times that of Q_9, the rates of biosynthesis of Q_9 can be estimated from the incorporation of presumably common precursors of isopentenyl pyrophosphate into coenzyme Q and cholesterol. Since the specific activity of cholesterol was twice that of coenzyme Q_9, the difference in absolute rates of synthesis was 40-fold. Lindstedt and Norman (1956) estimated, from studies of cholesterol turnover in the rat, that 1·5 mg. of cholesterol is synthesized per day by rat liver. On this basis, the rate of coenzyme Q_9 biosynthesis by rat liver would be approximately 40 μg./day or 1·5 μg./hour.

Table VIII

SYNTHESIS OF COENZYME Q_9 BY LIVER SLICES *in vitro*

Experiment No.	Radioactivity of [2-^{14}C]mevalonate μc/g. liver	Radioactivity of products: Coenzyme Q_9 fraction (R_F = 0·38) c.p.m.	"Fast moving fraction" (R_F = 0·81) c.p.m.
1	3·1	420	7,800
2	3·1	490	8,500

* *Conditions*: each beaker contained 1·5 g. of liver slices in 10 ml. of Krebs-bicarbonate medium. Mevalonate was present in a concentration of 2 m-mol/l. Gas phase: 5 per cent CO_2, 95 per cent O_2. Time of incubation at 38°, 3 hours. Tissue from four beakers pooled for isolation of coenzyme Q in each experiment. The fraction from alumina was chromatographed on paper impregnated with Vaseline with *n*-propanol : water, 4:1 and the R_F values refer to that system.

As additional evidence that rat liver can effect the synthesis of coenzyme Q from an isoprene precursor, [2-^{14}C]mevalonate incorporation into coenzyme Q_9 in rat liver slices was demonstrated. The results of two typical experiments are shown in Table VIII. After incubation in the Dubnoff shaker, the slices were washed, extracted with Bloor's reagent, and the coenzyme Q fraction isolated on alumina as previously described. When this portion was chromatographed on paper impregnated with Vaseline, using *n*-propanol : water, 4 : 1, the data shown in Table VIII were obtained. Although the "fast-moving fraction" contained most of the radioactivity, by elution of the Q_9 fraction

and rechromatography, constant specific radioactivity corresponding to 800 c.p.m./mg. of initial Q_9 could be demonstrated. The final product was recrystallized without loss of radioactivity with authentic coenzyme Q_9.

With regard to the contribution from phenylalanine *in vivo*,

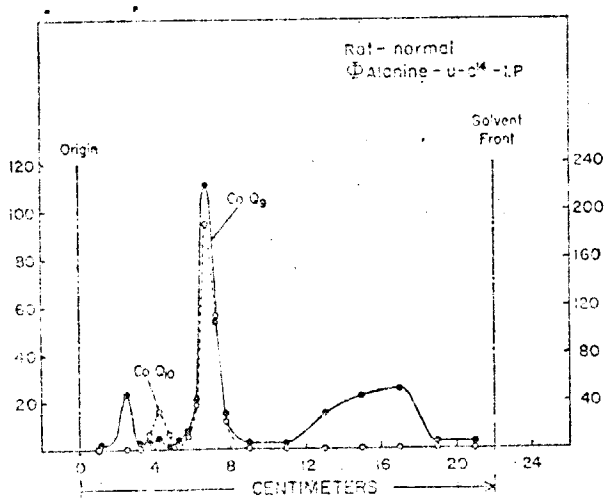

FIG. 8. Radiochromatogram of coenzyme Q fraction from normal rat given [U-^{14}C]phenylalanine intraperitoneally, after purification on alumina. Vaseline-impregnated paper was used and the chromatogram developed with *n*-propanol:water, 4:1. The solid line represents radioactivity in scintillation c.p.m. The dotted line represents concentration of coenzyme Q (µg.) determined spectrophotometrically.

the radiochromatogram obtained for the [U-^{14}C]phenylalanine-labelled coenzyme Q_9 fraction from alumina is shown in Fig. 8. This again shows predominant labelling of Q_9, although there are other radioactive peaks present in this chromatogram which are not identical with those seen in the coenzyme Q fraction obtained from alumina after [1-^{14}C]acetate administration. Since the radioactivity appearing in the coenzyme Q_9 from [U-^{14}C]-

phenylalanine could have resulted from the recycling of acetoacetate obtained during the catabolism of phenylalanine, the incorporation of radioactivity from phenylalanine into coenzyme Q_9 did not constitute definitive evidence for the direct incorporation of carbon from the phenylalanine ring into the coenzyme ring.

In order to obtain additional evidence on this point, the diacetate of radioactive dihydrocoenzyme Q_9 was made according to the procedure of Lester and co-workers (1959) and recrystallized from ethanol (m.p. 37°; $\lambda_{max.}^{ethanol}$ 268 mµ ($E_{1cm}^{1\%}$ = 7·09). Various degradations of this derivative were then attempted with permanganate (Olson, Dialameh and Bentley, 1960). Direct oxidation of 200 mg. of dihydrocoenzyme Q_9 diacetate gave a mixture of highly oxidized fragments from which no recognizable ring compound could be isolated. In view of this discouraging development, a series of Kuhn-Roth degradations was carried out on radioactive dihydrocoenzyme Q_9 diacetates derived from [1-^{14}C]acetate and [U-^{14}C]phenylalanine, to gain information about the distribution of radioactivity in the lateral methyl-containing α-carbon moieties of the side chain. This procedure also captures one mole of acetic acid from the ring (the 5-methyl group and its adjacent carbon atom), and these carbons also appear in the product isolated. The acetic acid, distilled from the chromic acid oxidation mixture, was chromatographed on celite with 2 N-sulphuric acid and oxidized further by the Schmidt procedure (Wolff, 1946). The carboxyl and methyl carbon atoms were then counted separately as $Ba^{14}CO_3$, corrected to infinite thickness and compared with a known standard. The results of these experiments are presented in Table IX. For the coenzyme Q_9 labelled with [1-^{14}C]acetate, the predicted labelling of the acetic acid obtained from the Kuhn-Roth procedure was found. Although the total recovery of radioactivity was less than anticipated (50 per cent for side chain labelling), similar difficulties were encountered by Wüersch, Huang and Bloch (1952) in the

study of distribution of radioactivity in the iso-octyl side chain of cholesterol biosynthesized from [1-^{14}C]acetate. The distribution of the radioactivity in the acetic acid isolated from [U-^{14}C]-phenylalanine is consistent with some recycling of a uniformly labelled acetoacetate—derived from this substrate—into mevalonate. The recovery of total radioactivity in this instance is also lower than calculated on the basis of a ring : side chain partition

Table IX

KUHN-ROTH DEGRADATIONS OF ^{14}C-LABELLED COENZYME Q_9 DIACETATES DERIVED FROM RAT LIVER

Substrate	Radioactivity recovered*		
	COOH μc/m-mole	CH_3 μc/m-mole	Total per cent
[1-^{14}C]acetate	0·024	0	29
[U-^{14}C]phenylalanine	0·008	0·007	18

* Radioactivity is adjusted to 1·0 μc/m-mole for the initial coenzyme Q_9 diacetate.

of radioactivity derived from the previously presented coenzyme Q_9: cholesterol specific activities. This calculation indicated that 34 per cent of the total radioactivity should be recovered. Although consistent with the hypothesis presented in Fig. 1, the results of the Kuhn-Roth degradations still do not constitute proof of direct incorporation of label from phenylalanine into the coenzyme ring.

Experiments are now in progress to elucidate this question. Ozonolysis of the dihydrocoenzyme Q_9 diacetate has been found by Morton and co-workers (1958) to give acetone, laevulinaldehyde and the aldehyde of the aromatic ring compound, 1,4-dimethoxy-3,4-dimethoxy-5-methylphenylacetaldehyde. Mild oxidation of these products will give the corresponding acids. Comparison of the activities of these fragments in coenzyme Q_9, biosynthesized from [1-^{14}C]acetate and [U-^{14}C]phenylalanine, should give the requisite information.

Summary

It has been shown that coenzyme Q_9 is the main form of the coenzyme Q in rat liver, and is biosynthesized from [1-^{14}C]acetate and [U-^{14}C]phenylalanine when these precursors are given parenterally to intact or enterectomized rats. Coenzyme Q_{10} is not synthesized by rat liver to any appreciable extent, and it appears in the faeces of rats fed coenzyme Q-free diets, suggesting that coenzyme Q_{10} may be mainly exogenous in this species. The pattern of labelling in coenzyme Q_9, which is observed after giving [1-^{14}C] acetate, is consistent with biosynthesis of the side chain *via* mevalonate and isopentenyl pyrophosphate. The pattern of labelling in coenzyme Q_9 which is observed after administration of [U-^{14}C]phenylalanine is consistent with partial direct synthesis of the ring fragment plus partial degradation and reincorporation of carbon in the isoprene side chain.

Acknowledgment

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DISCUSSION

Birch: With regard to the question of the origin of the ring, I would agree that something like toluquinol is the precursor. The question is whether it arises from acetate or from something related to tyrosine or phenylalanine. It seems to me that both routes are not necessarily excluded, particularly in different organisms. We were quite astonished to discover that in *Penicillium urticae* we get gentisic acid from methylsalicylic acid, which is an acetate-derived compound; we expected it to come from tyrosine. Although I agree that a route through homogentisic acid could be a possibility, I think certain further experiments are called for. These would involve incorporation of a specifically labelled precursor and examination of the product to see

precisely where the label is to be found. Mere incorporation of label into a product can be deceptive. The obvious experiment would be to use phenylalanine labelled in the 3-position of the side chain, to see whether or not the methyl on the ring is specifically labelled.

Olson: We have such an experiment in progress. In another experiment, the preliminary results on ozonolysis of the [U-^{14}C]phenylalanine labelled coenzyme Q_9 has shown the ring fragment, i.e. a phenylacetic acid derivative, to be four times a shot as the initial material. The results with [3-^{14}C]phenylalanine will be of particular interest.

Birch: Did you try toluquinone?

Olson: We have injected toluquinone into rats at doses of 20–100 mg./100 g./day and have observed acute toxicity terminating in death within a few hours at the higher doses, and no effect upon hepatic coenzyme Q levels at the lower doses over periods up to 7 days. ^{14}C-labelled toluquinone should also be examined for evidence of incorporation into coenzyme Q. We have studied the effect of diets deficient in phenylalanine upon the coenzyme Q content of liver and have found no significant effects. We must remember, however, the relatively large reservoir of free amino acids which exists, even in the deficient animal, in relation to the relatively slow rate of biosynthesis of the coenzyme.

Rudney: Since Dr. Wiss finds very little incorporation of phenylalanine, there appears to be a disagreement with Prof. Olson's data. I wonder whether this difference reflects a difference in experimental conditions.

Wiss: Using ^{14}C-acetate as a precursor, Prof. Olson found that the intestinal synthesis of ubiquinone (50) interferes with that in the liver. We could not find such an interference when ^{14}C-mevalonic acid was administered. The incorporation of acetate into the liver ubiquinones is rather poor, about 1000 times less than that of mevalonic acid. The interference of the bacterial synthesis in the case of acetate may be explained by assuming that its incorporation into ubiquinone (50) by the intestinal bacteria is higher than by the liver.

Olson: In answer to Dr. Rudney, the incorporation rates for phenylalanine into coenzyme Q in rat liver by Dr. Wiss and ourselves are about the same, i.e. 0·001 to 0·002 per cent of the administered dose.

With acetate our incorporation rate is between 0·003–0·006 per cent, which is, of course, much lower than that observed with mevalonate. Dr. Wiss, what was the duration of the experiment in which you studied the labelling of ubiquinone (50)?

Wiss: Two hours.

Olson: We had the idea that the absorption of a fat-soluble factor in intestinal micro-organisms which would have to be admitted to the body by way of the usual fat-absorption mechanisms, including transfer through the gut, distribution in the chyle, and uptake by the liver, would take longer than 1–2 hours. The fat-tolerance curve extends over 4–6 hours. Your initial experiment lasted four days, I believe, Dr. Wiss, in which time this type of mechanism would have time to operate and provide entry of microbiologically synthesized coenzyme Q to the liver. With the 2-hour experiment I find some difficulty in seeing this latter process occur as rapidly.

Morton: Glover and Lawson, in my laboratory, took the whole intestine, washed it clean, everted it and incubated it with mevalonate or acetate in the presence of a few mg. of unlabelled ubiquinones. In these *in vitro* experiments they got incorporation into ubiquinones (45) and (50).

Olson: This may indicate that the ubiquinone (50) is coming from the tissue of the gut and not the flora.

Morton: That is possible. Glover eliminated the bacteria, and also eliminated transport from other sites.

Olson: We may have eliminated a much more active tissue than the liver. We were preoccupied with the liver because we were intent on comparing these studies with other ones involving the action of α-tocopherol on the liver. In fact, we may have missed the most active tissue in which to study biosynthesis of coenzyme Q.

Morton: The everted sac is a very active tissue.

Olson: In our most recent studies using 500 μc of [1-^{14}C]acetate, we find low, but significant, labelling of hepatic ubiquinone (50). This could have come from the gut tissue *via* the portal circulation.

Arnstein: With reference to the phenylalanine experiments, it might be quite interesting to try the effect of *p*-fluorophenylalanine, since this analogue would probably inhibit the hydroxylation of phenylalanine.

Olson: Yes, we were looking for good blockers; it appears almost impossible to block this process nutritionally.

Kon: Prof. Olson, when you consider that microbial synthesis might be involved, do you postulate that there might be direct absorption or that it would be by recycling of faeces?

Olson: In the rat it could be either.

Kon: If it were by recycling of faeces you could check that by putting the rats in the special tubes that prevent coprophagy.

Olson: The only issue in our minds is whether or not the faeces contribute coenzyme Q_{10} in the rat.

Moore: You were unable to prevent liver necrosis with ubiquinone which, after all, is rather like vitamin A, and yet you found you could prevent it with selenium. What has selenium got that ubiquinone has not?

Olson: This is an important problem for further study. We have shown that selenium will increase the level of certain sulphur-containing compounds in the liver (1958, *Fed. Proc.*, 17, 498). The most dramatic one is coenzyme A. The effect on glutathione, which is depleted by sulphur restriction, is not affected as dramatically by selenium as is coenzyme A. We believe that there is some inter-relationship between selenium and sulphur in metabolism. This somehow crosses over the antioxidant function of α-tocopherol. We believe that the α-tocopherol is not itself participating in electron transport, a view already expressed by Prof. Slater, but that it stabilizes the lipoproteins of the mitochondria and of the cell membrane. The lack of stability in the liver cell membrane could contribute to the necrosis. The exact inter-relationship between selenium, sulphur and vitamin E is still an interesting problem for future research.

Crane: Have you done any experiments where ethionine has been tried in animals and then the Q isolated to see if ethoxyquinones come out?

Olson: No; we have some choline-deficient animals in the freezer, and we are going to study them to see whether hepatic coenzyme Q levels fall. Transmethylation should be involved in the formation of the methoxyl groups.

Some peculiar observations have been made about depressed respiration in choline-deficient animals. Liver slices corrected for lipid from

choline-deficient animals do not respire as actively as normal liver tissue. It has been reported further that cytochrome-lipid complexes can be found in the cytoplasm of the fatty liver and that the mitochondria are altered when viewed in the electron microscope. In heart muscle too, in choline deficiency, for some unexplained reason there is a decrease in Q_{10}. We wondered whether this might not be due to the limitation of labile methyl for O-methylation in synthesizing coenzyme Q.

Crane: This would fit in very nicely with the rate of labelling per mole of the substituents other than methoxyl.

Olson: I think that if one uses different labels for the molecule put together from so many parts, such as coenzyme Q, one need not worry too much about the gross inter-relationships of the rates of labelling of these various precursors. It is undoubtedly true that the rate of labelling of a given molecule is as much dependent upon the dose of compound administered and the pool sizes of the intermediates lying between the compound administered and final molecule synthesized as upon the rates of transformation of these metabolites. It would be interesting, however, to obtain a time curve of changes in specific activity of the various precursors of coenzyme Q.

Bredie: Is anything known about the biosynthesis of the naphthoquinone ring? Information regarding the biosynthesis of quinones might be obtained with the aid of bacterial mutants. This approach has certainly contributed to our understanding of many of the other biosynthetic pathways of the cell.

Birch: It might be an acetate-derived compound, but there are not enough oxygens in it to tell by looking at it. No tracer work has been done on it to my knowledge.

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Studies on Coenzyme Q

PATTERN OF LABELING IN COENZYME Q_9 AFTER ADMINISTRATION OF ISOTOPIC ACETATE AND AROMATIC AMINO ACIDS TO RATS*

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The isolation of coenzyme Q_{10}[1] from the tissues of mammals by Morton at Liverpool (1) and by Lester, Crane, and Hatefi at Madison (2), and the demonstration of its role in electron transport (3) have created great interest in its biochemical origin. Despite the close structural relationship of this compound (4, 5) to the fat-soluble vitamins E and K, it appears that, unlike these vitamins, coenzyme Q is synthesized in the mammal from relatively simple precursors. Our interest in the biosynthesis of coenzyme Q stemmed from our failure to demonstrate any appreciable fall in hepatic coenzyme Q levels in young rats fed diets devoid of coenzyme Q and the other fat-soluble vitamins for appreciable periods of time (6). On similar diets, α-tocopherol levels in liver could be demonstrated to fall to low levels. Morton (1) and Moore (7) have made similar observations. These findings suggested that coenzyme Q can be synthesized in the tissues of the rat although the possibility of intestinal synthesis by the microflora with subsequent absorption of the compound as in the case of vitamin K remained open.

During the past several years, we have carried out an extensive experimental program to elucidate the biosynthesis of CoQ in the rat under various conditions, including those minimizing the contribution of the intestinal microflora. At the outset (6) it seemed likely in mammals that the isoprenoid side chain was derived from acetate via the now well established pathway for isoprenoid biosynthesis via mevalonate and that the benzoquinone moiety came from a preformed essential aromatic nutrient such as phenylalanine. In this paper we describe the isolation of CoQ_9 and Q_{10} from rat liver and report the results of studies of the incorporation of acetate-1-^{14}C, phenylalanine-U-^{14}C, phenylalanine-3-^{14}C, tyrosine-U-^{14}C, tyrosine-3-^{14}C, and acetate-2-^{14}C into one or both of these coenzymes in rat liver. The distribution of the radioactivity between the aromatic nucleus and the side chain in CoQ labeled with ^{14}C from these precursors was also determined by a semimicro chemical degradation of the radioactive CoQ developed in this laboratory (8). The procedure depends upon low temperature ozonolysis followed by neutral permanganate oxidation of the resulting aromatic aldehyde. Levulinaldehyde as the bis-2,4-dinitrophenylhydrazone was isolated as a product of the side chain. Preliminary reports of these results have appeared (8–12).

EXPERIMENTAL PROCEDURE

Animals—Young male albino rats of the Sprague-Dawley strain (Charles River Breeding Laboratories, Brookline, Massachusetts) weighing 150 to 250 g were used in these experiments. The stock diet was Purina chow which contained 25% protein (N × 6.25) and 5% fat. Purified diets containing 18% casein and 6% lard were also used as indicated (13). Kilogram lots of rat liver for the isolation of CoQ_9 and Q_{10} were obtained from stock animals on ordinary chow diets. The nature of the diet fed to the animal did not appear to influence the concentration of CoQ or the distribution of CoQ homologues in the liver. In given experiments designed to demonstrate the biosynthesis of CoQ in the absence of the microflora of the intestinal tract, the whole small and large intestine from the first portion of the duodenum to the rectum was removed surgically under Nembutal anesthesia (50 mg per kg). The operation involved the ligation of the portal vein, splenic and mesenteric arteries, resection of the spleen, section of the duodenum between ligatures below the point of entry of the common bile duct, and finally section of the rectum just above the anus. The whole intestinal tract was then removed by blunt dissection and the abdomen closed. Without intervention such animals were observed to survive from 4 to 12 hours.

Vitamin A deficiency was induced in some of the animals used in these experiments in order to increase the incorporation of precursor radioactivity into hepatic CoQ_9 (14). Weanling rats 50 to 60 g in weight were fed the diet of Mayer and Krehl (15) for 2 to 4 weeks at which time they weighed 140 to 180 g and had approximately doubled their hepatic CoQ content.

Chemicals—All solvents were redistilled with the exception of analytical grade diethyl ether which was peroxide-free. All reagents were analytical grade. All isotopic substrates but the β-labeled aromatic acids were obtained from Nuclear-Chicago Corporation, Desplaines, Illinois. The acetate-1-^{14}C and acetate-2-^{14}C had specific activities of 2.0 mc per mM. The L-phenylalanine-U-^{14}C and L-tyrosine-U-^{14}C had been isolated from *Chorella* which had been grown in a medium enriched with

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[1] The abbreviations used are: CoQ, coenzyme Q; CoQ_n, coenzyme Q_n with n denoting a specified number of isoprene units in the side chain; phenylalanine-U-^{14}C and tyrosine-U-^{14}C refer to uniformly or randomly labeled phenylalanine and tyrosine, respectively.

^{14}C and were isolated after hydrolysis by ion exchange chromatography and crystallized to constant specific activity. Their radiochemical purity was attested to by carrier dilution with known authentic L-phenylalanine and L-tyrosine, respectively, by paper chromatography in 1-butanol-water-acetic acid, and by paper electrophoresis. The specific activity of the L-phenylalanine was 10.6 mc per mM; that of L-tyrosine was 10.0 mc per mM. DL-Phenylalanine-3-^{14}C and DL-tyrosine-3-^{14}C were obtained from New England Nuclear Corporation, Boston, Massachusetts. These compounds were unequivocally labeled in the β-carbon atom by the following procedure (16).[2] A Grignard condensation of the appropriate phenyl magnesium bromide with $^{14}CO_2$ was carried out to obtain the corresponding benzoic acid. The carboxyl of this acid was then reduced with lithium aluminum hydride to the corresponding alcohol which was then brominated and allowed to react with ethylacetamidocyanoacetate in the presence of sodium ethoxide. The condensation product was then hydrolyzed to yield the desired ^{14}C-β-labeled amino acid and crystallized to constant specific activity. The specific activity of the DL-phenylalanine-3-^{14}C used was 4.4 mc per mM; that of DL-tyrosine-3-^{14}C was 10.6 mc per mM. These substrates were administered to rats without isotopic dilution as specified.

General Procedure for Isolation of Crude CoQ by Alumina Chromatography—The animals were killed by decapitation and the livers quickly removed into 10 volumes of Bloor's reagent (alcohol-ether, 3:1), and homogenized with a high speed Sonifier for 2 minutes at room temperature. The extract was then filtered and the precipitated protein washed with an additional [illegible] volumes of Bloor's reagent. The extract was then evaporated [illegible] damp dryness in a rotary vacuum evaporator at 45°. KOH in methanol, 10%, was then added in the amount of 5 ml per g of initial tissue, and the extract was saponified in the presence of 1.2% pyrogallol for 2 hours at 80°. After dilution with an equal volume of water, the mixture was extracted three times with 2 volumes of petroleum ether. The petroleum ether extract was washed with water until alkali-free and then dried with Na_2SO_4 at 4°. The clear extract was then evaporated to dryness under reduced pressure and weighed.

The nonsaponifiable fraction was then purified by alumina chromatography according to the method of Heaton, Lowe, and Morton (17). Neutral alumina (Woelm) Brockman grade III (6% water) was used. The size of the column varied with the size of the sample. For a nonsaponifiable fraction from 1 to 5 g of tissue, a column 12 × 60 mm containing 3 g of alumina (Woelm) Brockman grade III was used. For 10 to 20 g of liver, a column 14 × 90 mm containing 5 g of alumina was used, and for large scale isolations columns 30 × 120 mm containing 30 g of alumina were used. A glass wool pad was pressed evenly into the tube to close its lower end. The alumina was suspended in 100 to 200 ml of redistilled petroleum ether (b.p. 40-55°), allowed to enter the column in a thin stream, and then was packed by washing with solvent. The sample was introduced in 5 to 15 ml of petroleum ether and the column developed with various solvents as indicated in Table I.

The fraction eluted with 4% of ethyl ether in petroleum ether [illegible]tained CoQ homologues, and it was purified by repeated [illegible]matography on alumina until "spectral purity" of the mixture of CoQ homologues in rat liver was obtained. Absorbances

[2] Details were kindly supplied by Dr. Seymour Rothchild, Technical Director of the New England Nuclear Corporation.

TABLE I

Description of fractions eluted from alumina column (10 g) in chromatography of nonsaponifiable fraction from rat liver

The nonsaponifiable fraction averaged 4 mg per g of rat liver. A usual nonsaponifiable fraction from 10 rats contained about 400 mg. The column was washed with 100 ml of petroleum ether before the sample was put on.

Fraction	Composition of eluent	Volume of eluent	Principal components of fraction
		ml	
1	Petroleum ether	100	Hydrocarbons
2	2% diethyl ether[a]	100	
3	4% diethyl ether	100	Coenzymes Q
4	6% diethyl ether	50	Isoprenoid alcohols and vitamin A
5	20% diethyl ether	150	Sterols
6	50% diethyl ether	100	Residue

[a] The diethyl ether was mixed with petroleum ether.

at $\lambda_{max}^{ethanol}$ 275 mμ of approximately $E_{1\,cm}^{1\%}$ = 180 and a change in absorption at 275 mμ upon reduction of the quinone with potassium borohydride ($\lambda_{O \to R}^{ethanol}$ 275 mμ) of $\Delta E_{1\,cm}^{1\%}$ of 155 were routinely obtained. The measurements of absorbance were carried out in a Beckman model DK-1 recording spectrophotometer. This spectrophotometric method was used as a basis of estimating the concentration of CoQ's during the isolation and purification of these quinones, with ($\lambda_{O \to R}^{ethanol}$ 275 mμ) $\Delta E_{1\,cm}^{1\%}$ of 142 for CoQ_{10} and 158 for CoQ_9. In the experiments in which radioactive CoQ fractions were obtained, they were purified to constant specific activity (as determined by counts per minute per $\Delta E_{1\,cm}^{1\%}$ (at 275 mμ) by repeated alumina chromatography.

In most experiments, the sterol-containing fraction from the alumina column was evaporated to a small volume, and taken up in 10 ml of acetone-alcohol, 1:1. Of 1% digitonin, 1.0 ml in 50% ethanol-water was added to precipitate cholesterol digitonide. This was washed with acetone-alcohol, 1:1, acetone, and ether and an aliquot taken for development of the Liebermann-Burchard chromogen according to Schoenheimer and Sperry (18). Another aliquot was decomposed with acetic anhydride and the supernatant sterol counted in the scintillation spectrometer. In a few experiments, the vitamin A present in Fraction 4 was rechromatographed on alumina to spectral purity ($\lambda_{max}^{ethanol}$ 328 mμ), $E_{1\,cm}^{1\%}$ = 1750, and counted for radioactivity as a negative control.

Paper Chromatography of CoQ Homologues—The semipurified CoQ fraction from alumina chromatography of constant spectral purity was then subjected to reverse phase paper chromatography in order to separate the homologues of CoQ present in rat liver. The system of Linn *et al.* (19) with Vaseline petroleum jelly-impregnated paper and *N,N*-dimethylformamide-water, 97:3, and the system of Lester and Ramasarma (20) employing siliconized paper and propanol-water, 4:1, were both used. The spots were visualized either by ultraviolet or by spraying with 1% permanganate. In both systems the presence of two homologues was shown as indicated in Fig. 1. Elution of the spots and spectrophotometric estimation of unstained chromatograms at 275 mμ as well as direct densitometric estimation of permanganate-stained spots showed rat liver to contain about 80% CoQ_9 and 20% CoQ_{10}. For preparative work, multiple

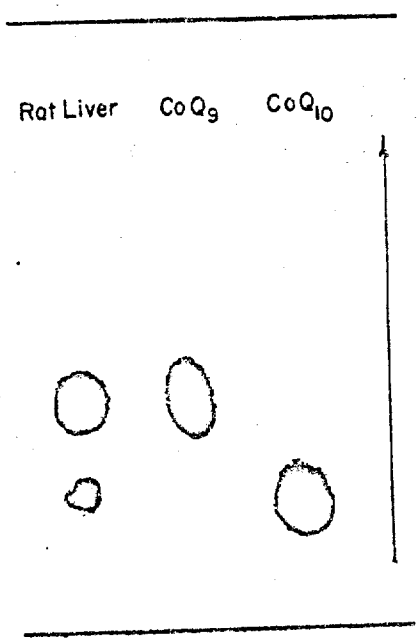

FIG. 1. Paper chromatogram of homologues of CoQ in rat liver. System is Vaseline petroleum jelly-impregnated paper developed with propanol-water, 4:1. Markers of synthetic CoQ_9 and CoQ_{10} in amounts of 10 μg each are shown. Spots are stained with 1% permanganate.

samples were spotted on long sheets of paper and the system changed to a hybrid of the two methods mentioned above, namely, Vaseline petroleum jelly-impregnated paper and propanol-water, 4:1. The advantage of this system was that the CoQ_9 and CoQ_{10} could be eluted separately from the paper strips and then be easily freed from other components of the system by rechromatography on alumina. The Vaseline petroleum jelly passed through the column with the petroleum solvent and the CoQ was eluted with 4% diethyl ether in petroleum ether. The purified CoQ_9 and CoQ_{10} were then assayed for radioactivity and crystallized with carrier to constant specific activity.

Chromatography on Polyethylene Powder—In order to separate the larger quantities of CoQ_9 and Q_{10} obtained from a total of 2 kg of rat liver, the crude CoQ fraction obtained from the alumina purification was subjected to further chromatography on polyethylene powder (21). Of polyethylene powder (Hostalen W), 40 g were suspended in 150 ml of acetone-water, 75:25, and poured into a tube to produce a column 2.2 × 23 cm. Of crude CoQ, 102 mg were dissolved in 150 ml of acetone-water, 3:1, and percolated onto the column. The column was washed with 1 liter of acetone-water, 75:25, and then 2-liter lots of 76 and 77% acetone, respectively. CoQ_9 was eluted with 3 liters of 81% acetone. The next fraction eluted with 83% acetone contained a 1:1 mixture of CoQ_9 and CoQ_{10}, and CoQ_{10} was eluted in essentially pure form by 1 liter of acetone-water, 85:15. The fractions containing only CoQ_9 and CoQ_{10}, respectively, were evaporated to dryness separately, taken up in petroleum ether, and rechromatographed on small alumina columns. The respective homologues were then eluted and twice recrystallized from methanol.

Preparation of Diacetates of CoQ_9 and CoQ_{10} Hydroquinones—The diacetates of CoQ_9 and CoQ_{10} were prepared by reductive acetylation with zinc and acetic anhydride according to the method of Lester *et al.* (22). The scale of the preparation varied from 5 to 50 mg, depending on the amount of material available. The reagents were varied in proportion to the amount of reactant. Both synthetic and natural CoQ_9 and CoQ_{10} isolated from rat liver were used. Usually 25 mg of CoQ were dissolved in 1 ml of acetic anhydride; 25 mg of zinc dust were added plus 0.2 ml of freshly distilled trimethylamine to combine with any free acetic acid in the anhydride. The mixture was warmed on the steam bath for 3 to 5 minutes and diluted with 5.0 ml of cyclohexane, filtered through glass wool, and transferred to a small separatory funnel. The cyclohexane phase was washed twice with 2.5 ml of 0.3 N HCl and three times with water. The solvent was distilled off in a vacuum and the semisolid residue dissolved in 3.0 ml of hot absolute ethanol. Crystals of the respective diacetates of CoQ_9 and Q_{10} hydroquinone formed in a few hours of standing at 4° and were filtered off after 24 hours. The respective derivatives from CoQ_9 and CoQ_{10} were recrystallized to constant melting points. In tracer experiments, the diacetates were recrystallized to constant specific activity. The diacetate of CoQ_9 hydroquinone melted at 37° and showed a single spot (R_F = 0.55) when chromatographed on a reverse phase paper chromatogram impregnated with Vaseline petroleum jelly and developed with propanol-water, 4:1. The diacetate of CoQ_{10} hydroquinone melted at 40° and likewise showed a single spot (R_F = 0.47) in the same reverse phase paper chromatographic system.

Measurement of Infrared Spectra of CoQ_9 and Q_{10}—The infrared spectra of synthetic CoQ_9 and Q_{10} and natural CoQ_9 from rat liver were compared in CCl_4 solutions by Dr. Hans Noll (23) of the Department of Microbiology, School of Medicine, University of Pittsburgh. He employed a Beckman model IR-4 spectrophotometer.

Isotopic Experiments—The radioactivity substrates were administered intraperitoneally to nonfasted rats in doses of 50 to 100 μc per rat. Ten to twenty animals were treated by injection in a single experiment and killed by decapitation 1½ to 3 hours later. In some experiments only the livers were pooled for the isolation of CoQ_9 and CoQ_{10}. In others the remaining carcass (devoid of head and intestinal contents) was also used. The incorporation of radioactivity into total carcass CoQ_9 in 3 hours was shown to be 5 or 7 times greater than that found in hepatic CoQ_9 at 1½ hours. The yield of CoQ in milligrams was also increased so that the average specific activity of the total carcass CoQ_9 was not appreciably different from that of the liver alone. This improvement in the recovery of radioactivity in CoQ_9 from the rat made chemical degradations of the isolated coenzyme feasible. In animals subjected to enterectomy, the isotopic substrates were administered intravenously and the animals killed after 90 minutes. CoQ_9 and CoQ_{10} were isolated only from the liver in these experiments.

Spectrophotometric estimation of the amount of CoQ present in the initial nonsaponifiable extracts was carried out with potassium borohydride as a reductant and at $\lambda_{S \to R}^{ethanol}$ 275 mμ, an $E_{1cm}^{1\%}$ of 158 as the constant. CoQ was isolated from liver and carcass and purified separately to constant specific radioactivity and spectral purity on Brockman grade III alumina as described above. At this point the two samples were combined and CoQ_9 (80 to 90% of the total CoQ) was isolated by preparative reverse phase paper chromatography, with paraffin oil-impregnated paper and 1-propanol-water, 4:1. Paper chromatography was repeated (at least once) to obtain constant

specific radioactivity of the CoQ_9. Rechromatography on alumina sufficed to remove the paraffin, and the spectrally pure CoQ_9 was then crystallized to constant specific radioactivity with 4 to 8 times its weight of authentic cold CoQ_9. The diacetate of CoQ_9 hydroquinone was then prepared by reductive acetylation as previously described (22). This derivative was also recrystallized to constant specific radioactivity, which was usually achieved in one recrystallization. The percentage of incorporation of administered label into CoQ_9 was calculated from the spectrophotometric estimation of amount of CoQ present in the initial nonsaponifiable extract and the specific activity of the finally purified diacetate of CoQ hydroquinone. This calculation overestimates the incorporation slightly because of the presence of the initial extracts of 10 to 20% of CoQ_{10} of lesser specific activity but this error is within the variability in biosynthetic rates found in duplicate experiments and is deemed sufficiently accurate for comparisons of the precursor substrates.

The radiochemically pure diacetate of CoQ_9 hydroquinone obtained after administration of given substrates was then degraded by ozonolysis to yield 3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid (m.p. 137°) as a ring fragment and the bis-2,4-dinitrophenylhydrazone of levulinaldehyde as a side chain derivative. Acetone from the terminal isopropylidene group was isolated as the 2,4-dinitrophenylhydrazone in preliminary experiments and found to agree in specific activity with that predicted from the levulinaldehyde moiety assuming consistent labeling of the side chain in agreement with established pathways for polyisoprenoid molecule biosynthesis (24). In the current experiments, the contribution of the acetone to the total activity of the CoQ_9 molecule was calculated on this assumption.

Liver and carcass cholesterol was isolated from the 20% diethyl ether-80% petroleum eluates of the alumina columns used for the isolation of CoQ. Appropriate aliquots were taken for estimation of total sterol by the method of Schoenheimer and Sperry (18) and for the estimation of radioactivity of the digitonin-precipitable sterol.

The radioactivity of cholesterol and CoQ and its derivatives was determined in toluene by liquid scintillation counting with diphenyloxazole as a phosphor. Samples were routinely checked for quenching and corrected if necessary. The bis-2,4-dinitrophenylhydrazone of levulinaldehyde was counted as an infinitely thick sample against a calibrated standard in a Nuclear-Chicago gas flow counter.

RESULTS

Isolation of CoQ_9 and Q_{10} from Rat Liver—The nonsaponifiable fraction from 2 kg of rat liver was chromatographed on a large column (110 × 25 mm) of 35 g of alumina Brockman grade III. The fraction eluted with 4% diethyl ether contained the yellow CoQ band which rechromatographed on alumina to spectral purity, *i.e.* $\lambda_{O \to R}^{ethanol}$ 275 mμ ($\Delta E_{1cm}^{1\%}$ = 155) and was recrystallized twice from methanol. Of mixed CoQ_9 and Q_{10}, 102 mg were obtained. Analysis of this mixture by paper chromatography showed that 81% was CoQ_9 and 19% was CoQ_{10}. This mixture was subjected to further chromatography on polyethylene powder and 40.2 mg of CoQ_9 and 5.7 mg of CoQ_{10} were obtained. The CoQ_9 melted at 44–45° (no depression with synthetic CoQ_9) and had the following spectral characteristics: $\lambda_{max}^{ethanol}$ 275 mμ ($E_{1cm}^{1\%}$ = 187); $\lambda_{min}^{ethanol}$ 236 mμ ($E_{1cm}^{1\%}$ = 30); $\lambda_{O \to R}^{ethanol}$ 275 mμ ($\Delta E_{1cm}^{1\%}$ = 159). This material showed one spot (R_F = 0.38) on a reverse phase paper chromatogram impregnated with Vaseline petroleum jelly and developed in 1-propanol-water, 4:1, identical with synthetic CoQ_9. It also chromatographed (R_F = 0.48) identically with synthetic CoQ_9 in the Vaseline petroleum jelly-diethylformamide system (19). The empirical analysis was satisfactory for

$C_{54}H_{82}O_4$

Calculated: C 81.56, H 10.39, CH_3O 7.81
Found: C[3] 81.43, H 10.14, CH_3O 8.32

The CoQ_{10} isolated from rat liver melted at 49° (no depression with authentic CoQ_{10}) and had the following spectral characteristics: $\lambda_{max}^{ethanol}$ 275 mμ ($E_{1cm}^{1\%}$ = 164); $\lambda_{min}^{ethanol}$ 236 mμ ($E_{1cm}^{1\%}$ = 22); $\lambda_{O \to R}^{ethanol}$ 275 mμ ($\Delta E_{1cm}^{1\%}$ = 142). This material showed one spot (R_F = 0.25) on a reverse phase paper chromatogram impregnated with Vaseline petroleum jelly and developed with propanol-water, 4:1. It was identical with synthetic CoQ_{10} in all systems tested. Empirical analysis was satisfactory for

$C_{59}H_{90}O_4$

Calculated: C 82.13, H 10.44, CH_3O 7.20
Found: C 81.98, H 10.64, CH_3O 7.26

Infrared Absorption of CoQ Homologues—Study of the infrared spectra of natural and synthetic CoQ_9 revealed them to be identical with each other and with spectra previously reported for CoQ_9 (21). Further analysis of the ratios of certain bands associated with the nucleus of CoQ_9 and CoQ_{10} to others associated with side chain functions revealed the type of pattern noted for the vitamin K homologues (23). These rates are presented in Table II. The side chain bands employed for the comparison are those at 2900 cm⁻¹ due to CH_2 stretching, at 1450 cm⁻¹ due to CH_2 scissoring, and at 1390 cm⁻¹ due to CH_3 bending. The nuclear functions employed were C=O and C=C stretching with bands at 1600 and 1650 cm⁻¹, respectively. The identity of the ratios for natural CoQ_9 isolated from rat liver and synthetic CoQ_9 is evident. Further, the homologous nature of CoQ_{10} is shown by the increase in the ratios for this member of the series.

TABLE II

Ratio of infrared absorptions at given pairs of wave numbers for synthetic and isolated Q_9 and Q_{10}

Preparation	2900: 1650 cm⁻¹	2900: 1600 cm⁻¹	1450: 1650 cm⁻¹	1450: 1600 cm⁻¹	1390: 1650 cm⁻¹	1390: 1600 cm⁻¹
Q_9 (rat liver)	1.09	1.26	0.59	0.68	0.37	0.43
Q_9 (synthetic)	1.14	1.35	0.59	0.68	0.37	0.44
Q_{10} (synthetic)	1.33	1.60	0.66	0.77	0.42	0.49

Preparation of Diacetates of CoQ_9 and CoQ_{10} Hydroquinones from Rat Liver—From 20 mg of CoQ_9 isolated from rat liver, 18.1 mg of the crystalline diacetate of CoQ_9 hydroquinone were obtained. The melting point was 36–37° (no depression with authentic CoQ_9 hydroquinone diacetate). It chromatographed as a single spot on reverse phase paper chromatograms indistinguishable from the synthetic CoQ_9 derivative. The natural diacetate of CoQ_9 hydroquinone also showed the typical absorption band in the ultraviolet, $\lambda_{max}^{ethanol}$ 238 mμ ($E_{1cm}^{1\%}$ = 8.7). The empirical

[3] Microanalyses were carried out by Dr. Carl Tiedcke, Teaneck, New Jersey, and by the Microanalytical Laboratory, Mellon Institute of Industrial Research, Pittsburgh, Pennsylvania.

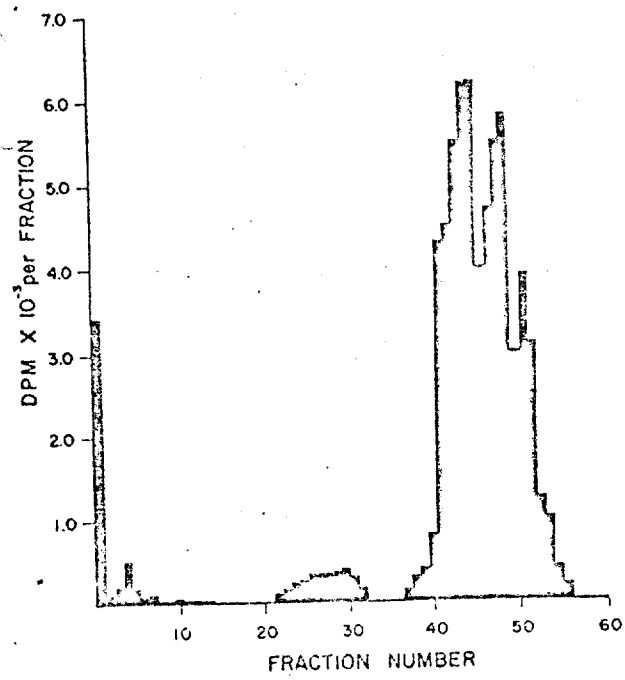

FIG. 2. Chromatography of nonsaponifiable fraction from livers of rats dosed with 100 μc of acetate-1-^{14}C. Each fraction represents 10 ml of eluate.

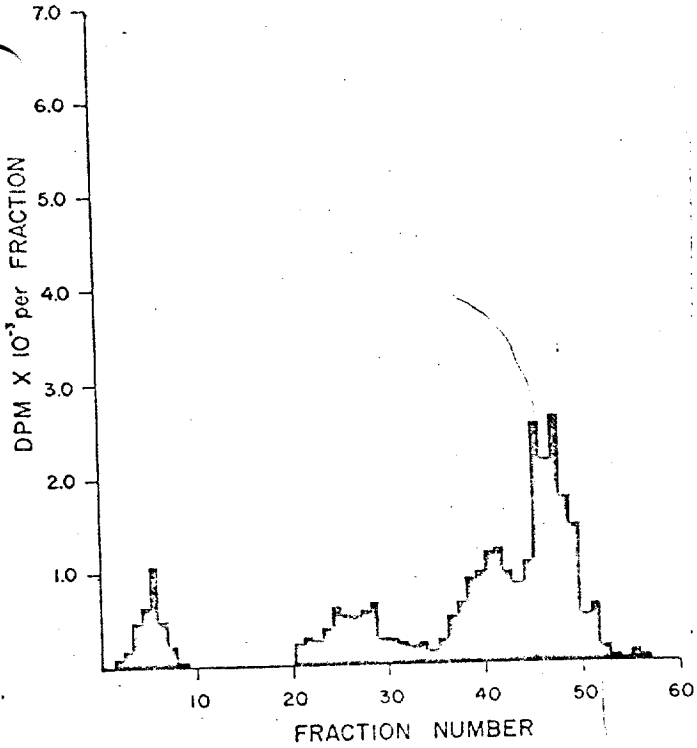

FIG. 3. Chromatography of nonsaponifiable fraction from livers of rats dosed with 100 μc of phenylalanine-U-^{14}C. Each fraction represents 10 ml of eluate.

nalysis of CoQ_9 hydroquinone diacetate was satisfactory for

$C_{58}H_{88}O_6$

Calculated: C 79.04, H 10.06, CH_3O 7.04, CH_3CO 9.76
Found: C 79.15, H 10.13, CH_3O 6.97, CH_3CO 9.83

From 4.1 mg of CoQ_{10} isolated from rat liver, 3.0 mg of the crystalline diacetate were obtained. The melting point was 40° with no depression with authentic CoQ_{10} hydroquinone diacetate; $\lambda_{max}^{ethanol}$ 268 mμ ($E_{1\,cm}^{1\%}$ = 6.8).

Incorporation of Radioactivity from Acetate-1-^{14}C and Phenylalanine-U-^{14}C into CoQ_9 and Q_{10} in Rat Liver—The nonsaponifiable fractions from the pooled livers of normal rats given either acetate-1-^{14}C or phenylalanine-U-^{14}C intraperitoneally in total doses of 100 μc and killed in 90 minutes were chromatographed on Brockman's grade II alumina. The recovery of radioactivity in 10-ml fractions eluted from the first alumina column, according to the schedule of Table I, is shown in Figs. 2 and 3 for animals receiving acetate-1-^{14}C and phenylalanine-U-^{14}C, respectively. Three major peaks of radioactivity are noted in each case. These peaks were associated with the hydrocarbons (Fractions 1 to 10), with CoQ homologues (Fractions 20 to 35), and with sterols (Fractions 36 to 55), respectively.

The incorporation of radioactivity into hydrocarbons and CoQ homologues was similar with the two precursors. The incorporation of radioactivity into the sterol fraction, however, was 3.5 times greater with acetate-1-^{14}C than with phenylalanine-U-^{14}C. The second hump of radioactivity appearing in the sterol fraction has not been associated with a discrete sterol. Most of the cholesterol is eluted in Fractions 35 to 45. Gas phase chromatograms of these two sets of fractions on columns with 2% silicone gum (SE 30) at 225° showed little qualitative change in the distribution of sterols. Cholesterol, small amounts of zymosterol, and traces of a C_{23} fraction occurred in both the sample containing Fractions 35 to 45 and in the one following composed of Fractions 45 to 55.

The change in specific radioactivity of the rat liver CoQ fraction derived from acetate-1-^{14}C upon subsequent purification on alumina is shown in Table III. More radioactive impurities are associated with CoQ fraction derived from acetate-1-^{14}C than with phenylalanine-U-^{14}C (6). This appears to be due to the presence of labeled isoprenoid alcohols associated with the CoQ fraction when acetate-1-^{14}C is the precursor (24). As indicated previously, the crude coenzyme fraction from rat liver was purified to a constant specific radioactivity as indicated by disintegrations per minute per $\lambda_{O \to R}^{ethanol}$ 275 mμ ($\Delta E_{1\,cm}^{1\%}$). The number of alumina chromatographies required to reach constant specific activity varied with the ^{14}C precursor used, more being required for acetate-1-^{14}C-labeled CoQ than that labeled with phenylalanine-U-^{14}C.

When crude ^{14}C-CoQ from rat liver of constant specific radioactivity and the spectral characteristics on alumina was subjected to preparative reverse phase paper chromatography with paraffin oil-impregnated paper and propanol-water, 4:1, and the CoQ_9 and CoQ_{10} spots eluted, CoQ_9 was found to have about the same specific activity as the crude CoQ, and CoQ_{10} was virtually nonradioactive. One-fourth of the radioactivity present in the crude CoQ sample was found in a leading fraction which showed no ultraviolet spectrum for CoQ. Reductive acetylation of the CoQ homologues obtained in this manner yielded the corresponding CoQ hydroquinone diacetate with a specific radioactivity usually within experimental error of that obtained for the purified CoQ. Occasionally, the derivative would show an appreciable drop in specific activity, indicating the necessity of making it the final proof of radiochemical purity. The purification of a typical sample is shown in Table IV. Appreciable losses in total radioactivity are sustained in obtaining a final radiochemi-

TABLE III

Changes in specific radioactivity of acetate-1-^{14}C-labeled CoQ fractions with purification

Procedure	Identity product	Amount	Total	Specific activity	Original counts
		mg	*d.p.m.*	*d.p.m./mg*	%
Alumina (1)[a]	Crude CoQ	3.10	255,000	82,200	100.0
Alumina (5)	CoQ_9 and CoQ_{10}	2.56	62,400	24,400	24.5
Paper chromatogram (1)	CoQ_9	1.75	40,400	23,200	15.9
Alumina (6)	CoQ_9	1.55	32,000	20,600	12.6
Derivative	CoQ_9 hydroquinone diacetate	1.05[b]	18,400	17,800[c]	7.2

[a] Number in parentheses refers to number of alumina chromatographies.
[b] Carrier CoQ_9 was added in the amount of 18.0 mg to make the derivative. Stated yield is calculated back to undiluted CoQ_9.
[c] Allowing for dilution of ^{12}C-carbon in diacetyl derivative, calculated specific activity per mg of original CoQ_9 was 19,900 or 96% of starting material. Additional recrystallization did not alter the specific activity and the derivative was considered radiochemically pure.

TABLE IV

Incorporation of radioactivity from acetate-1-^{14}C and phenylalanine-U-^{14}C into various lipids by rat liver in vivo in 90 minutes

Substrate	Rats[a]	Dose	Radioactivity of isolated products				Ratio of CoQ_9 activity to cholesterol activity
			Coenzyme Q_9	Coenzyme Q_{10}	Cholesterol	Fatty acids	
		μc/rat[b]	*d.p.m./mg*				
1. Acetate-1-^{14}C	N	50	1,500	60	2,920	1,450	0.51
2. Acetate-1-^{14}C	N	50	1,800	150	2,340	920	0.77
3. Acetate-1-^{14}C	E	500	19,900	330	74,200	22,050	0.27
4. Acetate-1-^{14}C	E	500	19,600	270	51,000	24,000	0.38
5. Phenylalanine-U-^{14}C	N	50	4,920	164	1,060	56	4.63
6. Phenylalanine-U-^{14}C	E	50	4,900	158	1,250	76	3.98

N, normal; E, enterectomized rats.
[b] Rats weighed 150 to 250 g and 2 to 10 rats were used in each experiment.

ically pure sample of CoQ_9. Parenthetically, it might be pointed out that coerystallization of radioactive CoQ_9 with cold CoQ_{10} carrier yielded a product whose specific activity changed very slowly with repeated crystallizations (14).

The distribution of radioactivity among CoQ_9, CoQ_{10}, cholesterol, and fatty acids isolated from the livers of rats dosed with either acetate-1-^{14}C or phenylalanine-U-^{14}C is presented in Table IV. Of the six experiments reported, three were performed in enterectomized rats. Vitamin A was isolated from the livers in two of the experiments in which acetate-1-^{14}C was given, purified chromatographically to spectral purity, and found to contain no radioactivity (6). The enrichment of radioactivity of all components measured was roughly proportional to the dose of given isotope administered. The incorporation of radioactivity into CoQ_{10} as compared with CoQ_9 was low, averaging only 3.5% of the specific activity of the more abundant homologue. Considering the difference in abundance, less than 1% of the radioactivity appearing in CoQ_9 appeared in CoQ_{10} in rat liver. The incorporation of radioactivity from acetate-1-^{14}C and phenylalanine-U-^{14}C into cholesterol and fatty acids reflected the extent to which these precursors generated acetyl-CoA and related small molecules. Acetate-1-^{14}C enriched both fatty acids and cholesterol effectively whereas phenylalanine-U-^{14}C was comparatively ineffective, particularly as regards the incorporation of its label into liver fatty acids. The ratio of specific radioactivity for CoQ to cholesterol averaged 0.48 for acetate-1-^{14}C and 4.31 for phenylalanine-U-^{14}C. This suggested that a dual pathway exists for the incorporation of label from phenylalanine into CoQ_9, permitting more label to enter the CoQ molecule than would be predicted from the isoprenoid pathway alone.

In order to validate a dual pathway for the biosynthesis of the ring and side chain components of CoQ, additional experiments were undertaken with vitamin A-deficient animals in which both liver and carcass were used as a source of coenzyme, and in which the isolated CoQ_9 hydroquinone diacetate degraded to obtain a fully substituted phenylacetic acid derivative as a ring fragment and the dinitrophenylhydrazone of levulinaldehyde as a derivative of the side chain.

The CoQ_9 concentration of livers from vitamin A-deficient animals was more than twice that of controls, *viz.* 276 ± 45[c] μg per g. fresh weight, for the vitamin A-deficient animals, and 108 ± 11 μg per g. fresh weight, for the controls (25). The concentrations of CoQ in the carcasses of normal and deficient animals were comparable and averaged about 20 μg per g of carcass (total body minus liver, head, and gastrointestinal contents). The carcass, however, supplied about 2.5 times as much CoQ_9 as the liver in the pooled samples of CoQ_9 subjected to study and degradation in these experiments. The amounts of CoQ found in the total nonsaponifiable fractions, the yield of crude CoQ from the alumina step, and the yield of CoQ_9 after two preparative paper chromatograms in seven isotopic experiments are presented in Table V. Approximately 50% of the CoQ_9 actually present was isolated after two paper chromatograms. The yield of carcass cholesterol ranged from 0.51 to 0.88 mg per g.

It may be seen from Table V that the radioactive CoQ_9 isolated

[c] Standard error of mean.

TABLE V

Incorporation of ^{14}C-labeled from selected precursors into coenzyme Q_9 and cholesterol in intact, normal, and vitamin A-deficient rats after 8 hours

Substrate	No. of animals[a]	Dose per experiment	Yields determined[b]	Amount of crude carcass CoQ isolated	CoQ_9 from paper chromatogram		Recrystallized CoQ_9	Recrystallized CoQ_9 hydroquinone diacetate	Incorporation into CoQ_9	Carcass cholesterol
		μc	mg	mg	mg	d.p.m./μM	d.p.m./μM	d.p.m./μM	%	d.p.m./μM
1. Acetate-1-^{14}C	10	1.0	40.4	25.2	13.4	4277	4300	4780	0.0150	4954
2. Acetate-2-^{14}C	10	1.0	46.8	31.1	21.6	3050	1770	1940	0.0060	2902
3. L-Phenylalanine-U-^{14}C	10	0.5	77.5	46.0	30.4	618	560	570	0.0053	251
4. L-Phenylalanine-U-^{14}C	10	1.0	37.4	27.5	15.9	2560	2850	2390	0.0050	518
5. DL-Phenylalanine-3-^{14}C	14	2.0	73.3	46.7	32.0	2440	2370	2420	0.0061	828
6. L-Tyrosine-U-^{14}C	10	1.0	42.2	36.3	16.1	7940	8550	9050	0.0220	666
7. DL-Tyrosine-3-^{14}C	10	2.0	62.2	43.5	29.6	2970	2880	2957	0.0052	2531

[a] Vitamin A-deficient rats were used throughout except in Experiment 5, in which normal rats were used.
[b] Total spectrophotometric estimate on nonsaponifiable fractions from liver and carcass.

TABLE VI

Specific radioactivity of rat body CoQ_9 hydroquinone diacetate and its degradation products after administration of given radioactive precursors

Substrate	Total dose of substrate	CoQ_9 hydroquinone diacetate[a]	Degradation products			Distribution of radioactivity		
			Phenylacetic acid[b]	Levulinaldehyde[c]	Acetone[d]	Ring fragment	Side chain[e]	Total
	mc	μc/mM × 10^2	μc/mM × 10^2			%		
1. Acetate-1-^{14}C	1.0	13.87	0.76	1.76	0.88	5.5	108.5	114
2. Acetate-2-^{14}C	1.0	8.43	0.19	1.07	0.71	2.4	109.9	112
3. L-Phenylalanine-U-^{14}C	0.5	2.99	2.64	0	0	88.0	0	88
4. L-Phenylalanine-U-^{14}C	1.0	8.30	5.92	0.32	0.19	71.5	33.5	105
5. DL-Phenylalanine-3-^{14}C	2.0	20.18	1.36	2.11	1.40	6.7	92.6	99
6. L-Tyrosine-U-^{14}C	1.0	38.20	4.93	0.30	0.18	70.2	36.9	107
7. DL-Tyrosine-3-^{14}C	2.0	24.20	1.44	2.63	1.75	5.9	91.1	97

[a] Dilution factor from body CoQ_9 due to addition of carrier ranged from 5 to 18.
[b] 3′,6′-Diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid.
[c] Measured as the bis-dinitrophenylhydrazone.
[d] Calculated from the activity of the levulinaldehyde.
[e] Side chain activity calculated by multiplying molar levulinaldehyde specific activity by 8 plus distribution of acetone.

after two paper chromatograms was radiochemically pure in most experiments. The specific activities reported in recrystallized CoQ, and the derivative are corrected for carrier addition. The agreement of the specific activity of the CoQ_9 recrystallized with carrier and the diacetate of CoQ_9 hydroquinone derived from this recrystallized CoQ_9 was within 10% in essentially all cases. Experiment 2 is an exception in which a radioactive impurity persisted through the paper chromatographic purification but was removed in the subsequent recrystallization with carrier and preparation of the derivative. This exception dramatizes the need for making a derivative of biosynthesized CoQ to be certain of its radiopurity.

The extent of incorporation of all precursors tested into total carcass CoQ_9 of the rat was of the same order of magnitude, the variation being from 0.005% with L-phenylalanine-U-^{14}C and L-tyrosine-3-^{14}C to 0.022% with L-tyrosine-U-^{14}C. L-Tyrosine-U-^{14}C was a considerably better precursor of CoQ_9 than L-phenylalanine-U-^{14}C and was slightly better in this series of experiments than acetate-1-^{14}C. DL-Tyrosine-3-^{14}C was only one-fourth as active a precursor of CoQ_9 as L-tyrosine-U-^{14}C, although the same differential incorporation rates were not noted for the corresponding radioisomers of phenylalanine. The incorporation of label into total body cholesterol was highest with the acetate-1-^{14}C and acetate-2-^{14}C, intermediate with tyrosine-3-^{14}C, and lowest with phenylalanine-3-^{14}C, phenylalanine-U-^{14}C, and tyrosine-U-^{14}C.

Table VI presents the results of degradation of the radioactive CoQ_9 hydroquinone diacetate isolated in each experiment. The radioactivity reported for the CoQ_9 hydroquinone diacetate is the radioactivity of the sample which was degraded. It may be seen that the substrates used fall into two categories, those which predominantly label the ring fragment and those which predominantly label the side chain. In agreement with previous experiments and hypotheses, acetate-1-^{14}C and acetate-2-^{14}C labeled predominantly the side chain with only 2 to 5% appearing in the ring fragment. The results indicate that between 70 and 88% of the radioactivity from uniformly labeled phenylalanine and tyrosine appears in the ring fragment and from 0 to 36% appears in the side chain. This is consistent with the two pathways of incorporation of aromatic amino acid carbon into CoQ, one proceeding by way of homogentisate to acetoacetate and then reincorporation via mevalonate into the side chain and the

er by direct incorporation of a nuclear fragment derived from e ring of phenylalanine and tyrosine into the ring of CoQ_9.

Most unexpected were the results obtained with DL-phenylalanine-3-^{14}C and DL-tyrosine-3-^{14}C. These substrates were chosen in order to demonstrate that the β-carbon of these two amino acids was incorporated into the methyl group of the ring fragment as expected from our initial hypothesis (6). The observation that very little radioactivity resided in the ring fragment when three labeled aromatic amino acids were given was not consistent with this previous hypothesis. It appears from these data that the β-carbon of phenylalanine and tyrosine appears only in the side chain of CoQ_9. It eliminates the possibility that the β-carbon of phenylalanine and tyrosine remains attached to the ring fragment in the conversion of the ring of the amino acid to the ring of CoQ_9.

The presence or absence of the gastrointestinal tract in these experiments (Table IV) did not in any way influence the rate of incorporation or distribution of radioactivity among the lipids analyzed. The biosynthesis of CoQ_9, therefore, is accomplished in the liver itself from metabolites available to it in the absence of the gut. In previous studies (6) it was shown that sterilization of the bowel with antibiotics likewise had no influence on the level or rate of incorporation of intraperitoneally administered precursors.

DISCUSSION

In 1959, Gloor and Wiss (14) reported that mevalonate-2-^{14}C incorporated into the crude CoQ of liver from normal and min A-deficient rats. This Q was isolated from liver, chromatographed on alumina and polyethylene powder, and crystallized with carrier CoQ_{10} to constant specific activity. From this questionable evidence, these workers assumed that the native material in the rat was CoQ_{10}. Subsequently, Gloor and Wiss (26) and Rüegg *et al.* (21) reported the isolation of CoQ_9 from the livers of vitamin A-deficient rats. We had independently observed that CoQ_9 was the main homologue in the normal rat (27) and experiments reported here confirm and extend the previous observation that CoQ_{10} is biosynthesized to only a limited extent in the liver of the rat from acetate-1-^{14}C. Wiss, Gloor, and Weber (28) found, on the contrary, that mevalonate-2-^{14}C administered either intraperitoneally or orally to rats resulted in 2 hours in the labeling of both hepatic CoQ_9 and Q_{10} in proportion to their concentrations in the liver. Although specific activities were not directly determined, it was inferred from these data that they were the same. In liver slices, we have noted only negligible incorporation of mevalonate-2-^{14}C into CoQ_{10}, whereas incorporation into CoQ_9 was excellent (29).

On the other hand, we have observed that CoQ_{10} isolated from *total carcass* of the rat 3 hours after administration of acetate-1-^{14}C is relatively more radioactive than that from the liver alone.[5] It is possible that the results of Wiss, Gloor, and Weber (28) can be explained on the basis of synthesis in the intestine, which has been noted by Lawson *et al.* (30), and transport to the liver. Since CoQ is found in the feces of rats on a CoQ-free diet (6), and since CoQ_{10} is absorbed and stored in the liver when administered to rats (6, 30), it is probable that a sizable fraction of hepatic CoQ_{10} in the rat is derived from the microflora of the gut.

It is of some interest that the rat and mouse are the only two mammals which have to date been shown to contain CoQ_9 as the predominant homologue. Examination of tissues from beef (3), human (19), horse (31), pig (32), and rabbit (19) have all indicated that CoQ_{10} is the only homologue present. In plants, both CoQ_9 and CoQ_{10} have been found (33). CoQ_{6-9} have been noted in microorganisms (2). The peculiar distribution of CoQ homologues characteristic of any species is no doubt the resultant of the distribution and activity of the enzyme systems which synthesize given elongated isoprenoid alcohol pyrophosphates for condensation with a ring moiety of CoQ, as well as the capacity of the intestine to absorb other CoQ homologues present in the intestine owing either to diet or to microbiological synthesis.

These results show clearly that acetic acid is incorporated into the isoprenoid side chain of CoQ_9 in the rat and does not contribute any carbon to the aromatic ring system. On the basis of labeling in the isoprenoid side chain only, the calculated incorporation of radioactivity from ^{14}C-acetate into the side chain of 3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid isolated as a ring fragment in the degradations carried out is 5.9% for acetate-1-^{14}C and 3.8% for acetate-2-^{14}C. The experimentally determined values of 5.5% and 2.4%, respectively, are in good agreement with this expectation. Gloor, Schindler, and Wiss (34) found labeled carbon from mevalonate-1-^{14}C exclusively in the side chain of CoQ_9 biosynthesized in the rat. These findings are consistent with the repeated observation that the benzene ring cannot be synthesized *de novo* from small molecules in the animal body and that nutrients containing preformed benzenoid nuclei are essential for the synthesis of such aromatic compounds as epinephrine, norepinephrine, thyroxine, and melanin. On the other hand, the biosynthesis of benzenoid substances via acetyl-CoA and malonyl-CoA is a recognized pathway in certain lower forms, notably fungi (35).

The incorporation of radioactivity from labeled phenylalanine and tyrosine into CoQ_9 is unequivocally established by these experiments. Wiss, Gloor, and Weber (28) reported that uniformly labeled phenylalanine orally administered to rats yielded only traces (0.001%) of radioactivity in the nonsaponifiable fraction from liver after 2 hours. In a later report (25) these same workers observed that when tyrosine of higher radioactivity was administered *per os* to rats, 0.2% of the administered dose was found in the nonsaponifiable lipids of the liver and 0.0008% of the dose was found in hepatic CoQ. No degradations of this biosynthesized CoQ were reported. In our experiments the extent of incorporation of radioactivity into the total nonsaponifiable fraction was 0.6% with acetate-1-^{14}C and acetate-2-^{14}C, 0.1% with L-phenylalanine-U-^{14}C, and 0.2% with L-tyrosine-U-^{14}C. The order of magnitude of incorporation of tagged acetate into CoQ_9, however, was the same as that observed with the aromatic amino acids. The ratio of specific activity per equivalent weight of isoprenoid carbon for CoQ-cholesterol was 1.5 to 8.0 for the uniformly labeled aromatic amino acids and 0.5 to 0.6 for acetate-labeled CoQ. This result conflicts with the observations of Wiss, Gloor, and Weber (25) who found that this ratio was 0.6 in liver lipids after administration of tyrosine-U-^{14}C to rats and 0.4 after mevalonate-2-^{14}C. Of more importance are the results of the chemical degradations of the radioactive CoQ_9 biosynthesized in the rat from this battery of precursors. Uniformly labeled phenylalanine and tyrosine contribute carbon to both the nucleus and the side chain of CoQ_9 whereas β-labeled phenylalanine and tyrosine contribute carbon *only* to the side chain. The small amount of radioactivity associated with the ring fragment isolated in these experiments corresponds to that

[5] R. E. Olson, unpublished observations.

found with acetate and may be assumed to be in the acetic acid side chain of the substituted phenylacetic acid. These findings eliminate a preformed toluquinone formed from homogentisate by decarboxylation as a precursor of the nuclear fragment in CoQ_9. Further, it opens up the unprecedented possibility that direct *C*-methylation of a benzenoid system may occur in animal tissues. In fact, preliminary studies in this laboratory (8) have shown that formate may be incorporated into the ring methyl group of CoQ_9 as well as into the *O*-methyls, the latter being well established by Glover *et al.* (36), Rudney and Sugimura (37), and Wiss, Gloor, and Weber (25).

One may well ask what the pathway is for the complete loss of the side chain from phenylalanine in the metabolism of its ring fragment to that of CoQ_9. The study of possible intermediates in our laboratory has led to the view that benzoate and *p*-hydroxybenzoate are on this pathway. The sequence is probably as follows:

Phenylalanine → tyrosine → *p*-hydroxybenzoate → Q nucleus → Q

Since the rate of incorporation of tyrosine into CoQ is approximately 4 times as fast as that of phenylalanine, it is reasonable to believe that tyrosine is more proximal to CoQ than is phenylalanine. The evidence implicating *p*-hydroxybenzoate as the next major intermediate has been presented in a preliminary communication (8). The evidence available from studies of the incorporation of carboxyl- and ring-labeled benzoate into CoQ_9 is consistent with that obtained from the two radioisomers of tyrosine in that the carboxyl group of benzoate is lost in its conversion to the coenzyme. Although it has been thought that reactions yielding phenols from tyrosine are generally attributed to the microorganisms of the gastrointestinal tract, recent evidence suggests that carbon-by-carbon chain shortening of tyrosine and phenylalanine is a physiological reaction. The conversion of isotopic phenylalanine to benzoate has been demonstrated in animals by Bernhard, Vuilleumier, and Brubacher (38), and in man by Grümer (39). According to the last author, the rate of this conversion is increased in phenylketonuria. The conversion of tyrosine to *p*-hydroxybenzoate has also been demonstrated in animals by Booth *et al.* (40). The mechanism of this chain shortening is at present obscure although Oates *et al.* (41) have demonstrated the conversion of phenylalanine to phenethylamine in patients with phenylketonuria. It is possible that decarboxylation followed by amine oxidation, typical of the metabolism of catecholamines, followed by α-oxidation of the resulting phenylacetic acid and ultimate decarboxylation is the sequence followed by tyrosine in generating the CoQ ring system.[6] The order of hydroxylations and alkylations (to yield the methyl group and the polyisoprenoid side chain) is, of course, unknown and is a subject for continuing study.

The over-all rate of CoQ_9 biosynthesis in the rat is slow. If one assumes, for the sake of an approximation, that total synthesis *de novo* of CoQ_9 occurs in its turnover in tissues, it is possible to approximate its rate of turnover from the relative rate of incorporation of acetate-1-^{14}C into the side chain of the coenzyme and into cholesterol, assuming that the isoprenoid pyrophosphates involved in both arise from a common pool. The specific activity of liver CoQ_9 biosynthesized from acetate-1-^{14}C was approximately one-half of that of simultaneously labeled hepatic cholesterol. The liver plasma pool of cholesterol in the rat, which is in isotopic equilibrium in 90 minutes, is approximately 20 times the size of the similar CoQ_9 pool in the rat. There is thus a 40-fold difference in the rate of incorporation of isoprenoid pyrophosphate molecules into the CoQ_9 and cholesterol pools. Lindstedt and Norman (42) estimated from studies of cholesterol turnover and excretion in the young adult rat that 1.5 mg of cholesterol are synthesized per day by rat liver. On this basis, the CoQ_9 biosynthesis by rat liver would be approximately 40 μg per day or 1.5 μg per hour. This very slow metabolic turnover is consistent with the low incorporation rates of isotope from both radioactive acetate and phenylamino acids into total carcass CoQ_9 of from 0.005 to 0.022% of administered isotope in 3 hours.

The contribution of intestinal flora to hepatic CoQ_9 biosynthesis seems negligible in view of almost identical results obtained in intact and enterectomized rats. The suggestion that an unknown aromatic fragment may be derived from the microflora of the gut (30) seems unlikely in view of the satisfactory incorporation of parenteral phenylalanine-U-^{14}C into hepatic CoQ_9 in the enterectomized rat.

[6] *Note*—It has since been established that *p*-hydroxyphenyllactic and *p*-hydroxycinnamic acids are intermediates in the chain-shortening process.

SUMMARY

Coenzyme Q_9 and coenzyme Q_{10} have been isolated from rat liver and characterized as the free quinones and as the respective coenzyme Q hydroquinone diacetates. Coenzyme Q_9 is the principal homologue in rat liver, making up 80% of the total coenzyme Q. ^{14}C-Labeled acetate, phenylalanine, and tyrosine are incorporated into total rat coenzyme Q_9 to the extent of 0.005 to 0.022% of the administered dose. Coenzyme Q_{10} is labeled much more poorly.

By chemical degradation of the ^{14}C-CoQ_9 hydroquinone diacetate obtained in these experiments the pattern of labeling of the coenzyme was ascertained. With acetate-1-^{14}C and acetate-2-^{14}C, radioactivity was found almost exclusively in levulinaldehyde derived from the side chain. With uniformly labeled L-phenylalanine-^{14}C and L-tyrosine-^{14}C, the radioactivity was found predominantly (70 to 88%) in the ring fragment, 3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid. The over-all incorporation of uniformly labeled tyrosine-^{14}C into CoQ_9 was 4 times greater than that observed with uniformly labeled phenylalanine-^{14}C. With DL-phenylalanine-3-^{14}C and DL-tyrosine-3-^{14}C, however, the incorporation was almost exclusively in the side chain.

These results are interpreted to signify that two distinct pathways exist for the incorporation of radioactivity from uniformly labeled phenylamino acid into CoQ_9 in the rat. One pathway involves the conversion of phenylalanine to tyrosine, homogentisate, and acetoacetate with reincorporation of the final product into mevalonate which serves as a side chain precursor. The other pathway involves complete loss of the side chain of the phenylamino acid in the process of generating a nuclear fragment which ultimately becomes the ring system of CoQ_9. The details of this latter novel pathway are under investigation.

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Utilization of salts of volatile fatty acids by growing sheep

31. Effect of frequency of feeding on the utilization of acetate and propionate by young growing lambs

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1. The effect, on the concentration of volatile fatty acids (VFA) in the rumen, of increasing the frequency of feeding from twice daily to four or six times daily was investigated in sheep [illegible] VFA salts. In a second experiment isocaloric rations containing [illegible] propionate were given to groups of lambs in two or six equal feeds daily. [illegible] which the lambs utilized the VFA salts was measured as gain in live weight, empty body weight and carcass weight and was compared to that of lambs receiving only [illegible]. The composition of the carcasses was estimated from that of the bin. 2. Increasing the frequency of feeding from twice daily to four or six times daily significantly reduced the [illegible] in rumen VFA composition associated with giving rations containing VFA salts [illegible]. In lambs receiving acetate and propionate, final live weight, empty body [illegible] were significantly greater than in lambs receiving only the basal [illegible]. The combustible energy from added VFA was apparently utilized more efficiently than the calculated metabolizable energy of the basal rations given above maintenance level, and there was no difference between the utilization of acetate and propionate. [illegible] significance between feeding twice daily and six times daily [illegible] groups of lambs [illegible] only the basal diet or between those given the diet [illegible] with VFA salts. Nor were there any interactions between VFA and frequency of feeding. 4. It is postulated that in ruminants the effect of frequency of feeding depends on the environment and level of feeding.

Previous experiments showed that additions of salts of volatile fatty acids (VFA) in two daily feeds caused marked changes in the concentration of VFA in the rumen content. These changes were associated with time of feeding and indicated a very rapid absorption of the VFA (Orskov & Allen, 1966). Balch & Rowland (1957) suggested that conditions might occur in which, owing to rapid absorption, the concentration of acetic acid in the blood might be so high as to cause wasteful oxidations. This led Orskov & Allen (1966) to suggest that the fate of VFA salts given in large quantities twice daily and that of VFA continuously infused might be different. They also suggested that the differences in results between their work and that of Armstrong & Blaxter (1957) and Armstrong, Blaxter, Graham & Wainman (1958), who applied continuous infusion, might be a result of such differences in techniques. Armstrong & Blaxter (1957) and Armstrong *et al.* (1958) found wide differences between the efficiency of the different VFA for lipogenesis whereas Orskov & Allen (1966) found no difference in the efficiency with which the energy from different VFA promoted gains in body tissues in lambs.

The experiments described here were a preliminary trial, in which it was confirmed

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that by increasing the frequency of feeding the marked changes in the rumen concentration of VFA associated with time of feeding could be reduced, and a feeding trial in which isocaloric quantities of acetate and propionate were given daily to groups of lambs in two or six feeds as additions to a basal ration of hay and concentrate. There were two control groups receiving the basal ration in two or six daily feeds. Gordon & Tribe (1952) and Rakes, Hardison, Albert, Moore & Graf (1957) showed that ruminants utilized their food with increased efficiency when the frequency of feeding was increased.

EXPERIMENTAL

Animals and facilities

Three mature wether sheep (Suffolk × Scottish halfbred) with rumen cannulas were used in Expt 1. They weighed approximately 130 lb each and were kept in digestibility crates.

In Expt 2, fifty-six Dorset Horn or Dorset Horn cross wether lambs were used. At the beginning of the experiment they were 10–14 weeks of age and weighed from 40 to 69 lb. The lambs were housed in a covered yard in individual pens bedded with sawdust.

Design and treatments

In Expt 1 the sheep were fed twice, four times or six times daily, according to a latin square design, on a diet consisting of hay and concentrate. The feeding periods were 10 days, during the last day of which rumen liquor samples were obtained. The feeding and sampling times are shown in Table 1.

A randomized block design was used in Expt 2. At the start of the experiment, lambs were allocated to blocks according to mean live weight on 3 consecutive days. The treatments were: treatment 1, initial slaughter group; treatment 2, basal diet (hay + concentrate) given twice daily to produce a gain of approximately 0·25 lb/lamb daily; treatment 3, basal (hay + concentrate) given six times daily; treatment 4, basal + acetate given twice daily; treatment 5, basal + acetate given six times daily; treatment 6, basal + propionate given twice daily; treatment 7, basal + propionate given six times daily.

The experiment was carried out during the summer of 1963. The initial slaughter group and the group fed twice daily were treated identically to the initial slaughter group and unimplanted groups used in a previous experiment (Orskov, Hovell & Allen, 1966, Expt 2). The composition of the hay and concentrate mixture and amounts of VFA salts added were as described by Orskov *et al.* (1966, Expt 2).

Management of the lambs

The food was weighed once daily and divided into the appropriate number of rations according to frequency of feeding. The feeding times in Expt 1 are shown in Table 1. The fistulated sheep ate all their rations in a few minutes except for one sheep which when receiving its food twice daily consumed the food in small quantities throughout the day. Results for this sheep at this time have been excluded from consideration.

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The lambs fed twice daily in Expt 2 were given their rations at 08.30 and 17.00 h, those fed six times daily were given food at 07.00 h, and every 2 h until 17.00 h. Uneaten food, if any, was weighed and removed daily. The weight of the concentrates and part of the hay, left uneaten in the initial part of the experiment were given to the same lambs later in the experimental period so that the required amount of concentrates and VFA salts was consumed by each animal. The animals fed six times daily frequently left small quantities of hay uneaten.

Table 1. *Expt 1. Times of feeding sheep receiving their daily allowance of food in two, four or six feeds daily, and times of removal of rumen samples*

Feeding time			
Six feeds daily	Four feeds daily	Two feeds daily	Sampling time
08.45	08.30	08.30	09.00
10.30	11.30	17.30	11.00
12.15	14.30	---	13.00
14.00	17.30	---	15.00
15.45		---	17.00
17.30	---	---	19.00

The lambs were dosed with an anthelmintic methylridine compound (Mintic; ICI) for intestinal worms, and no clinical signs of infestation occurred. Footrot was controlled by paring infected feet and by treatment with a chloromycetin tincture. Two lambs died as a result of urinary calculi. Apart from these incidences, the health of the animals was excellent.

Feeding levels

The sheep with rumen fistulas in Expt 1 received daily 1·88 lb hay and 2·63 lb concentrate, of which sodium and calcium acetates made up 50%. It was considered valid to use acetate to study the effect of frequency of feeding on the VFA concentrations in the rumen liquor as the three VFA salts were found to have similar effects on the chemistry of the respective acid in the rumen (Orskov & Allen, 1966).

To achieve the required growth rate, the animals receiving the basal control diet in Expt 2 were given 0·19 lb concentrate and 0·13 lb hay per 10 lb live weight until they weighed 50 lb. Above this weight, the daily ration was increased by 0·13 lb concentrate and 0·09 lb hay per 10 lb live weight. Those animals receiving the VFA salts were given the same basal diet with the addition of acetate or propionate. The lambs were weighed once every 2 weeks and food intake was adjusted accordingly.

Slaughter procedure and analysis

The initial slaughter group was killed before the experiment began. The lambs receiving the experimental diets were slaughtered over a 4-day period after 105–106 days on experiment.

After slaughter the weights of mesenteric fat and G in the omentum, of the pelt and of the alimentary tract, full and empty, were recorded. The carcasses were weighed between 1 and 2 h after slaughter. After 24 h chilling, the loins of the left sides were

removed as described earlier (Orskov *et al.* 1966). The final live weight was taken as the mean of the live weights at slaughter and 2 days before slaughter. Empty body weight was calculated as live weight at slaughter minus gut contents. The carcasses were graded by an official grader of the Ministry of Agriculture, Fisheries and Food.

Analysis of rumen liquor for VFA

The rumen liquor was collected into an Erlenmeyer suction flask using reduced pressure. The samples of rumen liquor were strained through muslin and centrifuged and of the supernatant fluid were then acidified with 0·5 ml concentrated orthophosphoric acid in a glass stoppered test tube. The subsequent procedure was similar to that described by Orskov & Allen (1966).

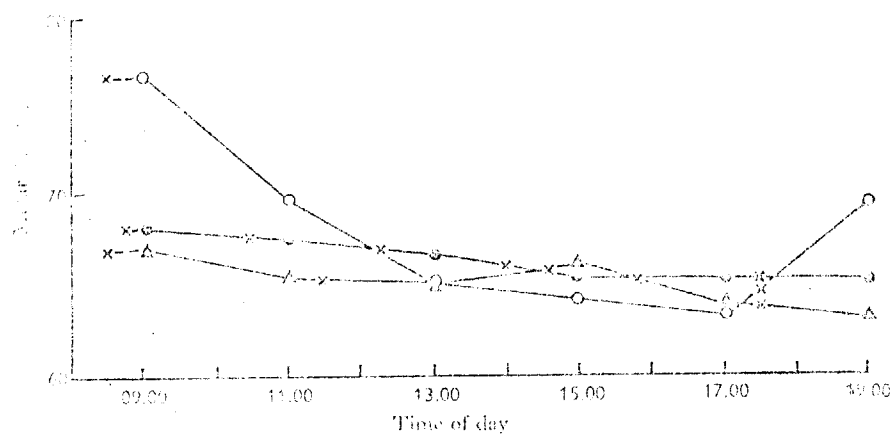

Fig. 1. Expt 1. Molar percentage of acetic acid in the rumen of sheep given a diet supplemented with acetate twice daily, ○—○, four times daily, △—△, or six times daily, ×—×. × shows feeding time.

Table 2. *Expt 1. Treatment means of log error mean square* (S^2) *and quadratic effect of the daily variation in the proportion of acetic acid resulting from a diet containing acetate and given to lambs in two, four or six feeds daily*

No. of feeds/day	Log S^2	Quadratic effect
6	0·4039	4·3
4	0·4548	3·5
2	1·2420	80·2
Standard error of difference between two means	±0·3192	±13·7

RESULTS

The results of Expt 1 are illustrated graphically in Fig. 1. The marked changes in the proportion of acetic acid associated with twice daily feeding with acetate were greatly reduced by increasing the frequency of feeding. The effects illustrated in Fig. 1 were substantiated by analysis of variance of the logarithms of the error mean

[illegible]

[illegible]

* Calculated by the method of Orskov (1966).

TABLE 4. *Expt 2. Treatment means of carcass and fat free carcass fat, empty body weight, carcass weight, fat and protein in the loin, and number of carcasses in each grade in the four treatment groups of the Ministry of Agriculture, Fisheries and Food, in groups of ewes 5 weeks slaughtered initially, or near the end of lambing, or six weeks later*

Treatment	Empty body weight (kg)	Carcass weight (kg)	Fat content (%)	Protein content (%)	Loin	No. of carcasses in each grade		
						A	B	C
Basal control	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Acetate	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Propionate	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Acetate + propionate	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Fat free carcass fat	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Protein	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Loin	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Number of carcasses in each grade	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

squares from each set of determinations of acetic acid (Table 2). The difference in variation between feeding twice and six times daily was significant ($P < 0.05$) and that between feeding twice and four times approached significance ($P < 0.1$). On [illegible] the linear and quadratic effects of the values from which the curves in Fig. 1 were constructed, it was found that the quadratic effect of feeding four or six times daily did not differ significantly from zero, whereas there was a highly significant quadratic effect when the animals were fed twice daily ($P < 0.001$).

The mean daily intake of hay and concentrate and VFA salts in Expt 2 calculated as [illegible] digestible energy (DE) is given in Table 3.

The most relevant results of Expt 2 are summarized in Table 4, where gains in [illegible] reflected in live weight, empty body weight and carcass weight are given, [illegible] with the composition of the loins and number of carcasses in each grade. [illegible] values were calculated for the two lambs which died and a third which at slaughter was found to be a cryptorchid.

There were no significant differences between the two frequencies of feeding nor were there any significant interactions between feeding frequency and VFA treatment.

The lambs receiving acetate and propionate grew faster and had a greater empty body weight at slaughter than those receiving the basal diet ($P < 0.001$) and, if the [illegible] are pooled according to diet, the differences between the basal control group and the VFA treatment groups combined in final live weight, empty body weight and carcass weight were significant at $P < 0.001$; there were no differences approaching significance between the acetate and propionate groups.

There were no differences that approached significance between the different groups in fat or protein content of loin. In the initial slaughter group, however, the carcasses had a lower fat percentage and a higher protein percentage than the groups receiving the feeding treatments ($P < 0.01$). The carcass gradings show that all the lambs produced satisfactory carcasses except the carcasses of the initial slaughter group which were graded lower than those of the other groups.

DISCUSSION

Influence of frequency of feeding of VFA salts on the rumen VFA composition

The results of Expt 1 demonstrated clearly that the marked changes in the composition of rumen VFA associated with giving the sheep VFA salts twice daily could be greatly reduced by increasing the frequency of feeding. Part of this reduced variation might have been an effect of less variation on the molar proportions from fermentation of the basal constituents of the food (Satter & Baumgardt, 1962; Bath & Rook, 1963). However, using a similar basal diet, Orskov & Allen (1966) found no appreciable diurnal variation when the basal diet was given twice daily, and it seems justifiable to assume that the reduced variation was a result of the salts being added to the rumen in smaller quantities at one time.

Influence of the frequency of feeding on the utilization of VFA as sources of energy

The results of Expt. 2 are in agreement with results obtained earlier (Orskov & Allen, 1966; Orskov *et al.* 1966). If the maintenance requirement is subtracted from the total intake, it can be calculated from data of Langlands, Corbett, McDonald & Pullar (1963) that the energy derived from acetate and propionate per kcal was utilized about 10% more efficiently to promote carcass gain than was the calculated ME of the basal diet. This estimate could be influenced by errors involved in calculating the maintenance requirements and the ME of the basal ration. However, if it is assumed that the utilization of ME increases with increasing level of concentrate in the diet (Blaxter, 1962), the results are in agreement with those of Orskov & Allen (1966) who showed that the utilization of VFA salts was equal to that of ME in concentrates.

Increasing the frequency of feeding did not influence the utilization of the VFA. The proposition made that the effect of twice daily feeding, with the resultant large increases in the rumen VFA concentrations, might have caused blood concentrations that exceed the metabolic capacity of body tissues must therefore be rejected. The results showed that the animals were able to utilize the VFA efficiently even when large increases in concentration occurred from twice daily feeding.

There were no signs of interactions between the utilization of VFA and feeding frequency. This suggests that the differences in the findings of Orskov & Allen (1966) and Orskov *et al.* (1966) and those of Armstrong and his colleagues (Armstrong & Blaxter, 1957; Armstrong *et al.* 1958) regarding the efficiency of utilization of VFA are probably not a result of difference in technique, namely feeding twice daily with VFA salts or using continuous infusions of the dilute acids. The difference in results will be discussed in a later communication.

Influence of feeding frequency on the efficiency of food utilization by ruminant animals

The effect of increasing the feeding frequency from twice to six times daily had little effect on the utilization of the basal diet. This finding led to a further inquiry into the observations of other workers where the influence of feeding frequency of isocaloric daily rations has been investigated. Table 5 presents a summary of the results of experiments on the effect of feeding frequency on daily live-weight gain when isocaloric daily rations have been given.

Except in a small scale experiment by Rakes, Lister & Reid (1961), the experiments in which improved gains were reported from increasing the frequency of feeding were all characterized by very low growth rates, and the only criterion for response was live-weight gain. In addition to the experiments in which no effects were noted from increasing the frequency of feeding, Rhodes & Woods (1962) in a series of experiments comparing different ratios of hay to concentrate and different combinations of feeding frequencies found no effect of increasing the feeding frequency beyond twice daily in animals making rapid gains. Kay & Hobson (1963), when reviewing the subject, concluded that ruminant animals benefit substantially from increased frequency of

feeding and that the results of Rhodes & Woods (1962) were an exception. This conclusion does not seem entirely justified. When moderate growth rates have been attained none of the experiments referred to has demonstrated a significant improvement in terms of live-weight gain when an isocaloric daily ration has been offered at intervals of greater frequency than twice daily. The results of the present work conform with these observations.

Table 5. *Summary of experiments in which the effects of frequency of feeding with isocaloric daily rations have been investigated*

Source of data	Type of feed	Animals	No. of feeds daily (a)	No. of feeds daily (b)	Growth rate (lb/day) (a)	Growth rate (lb/day) (b)
[illegible] (1932)	Hay and concentrate	Lambs	1	8	0·03	0·18
[illegible] Davidson, Albert [illegible]	[illegible]	Heifers	2	10	0·49	0·04
[illegible] Thomas & [illegible]	Hay and concentrate	Steers	2	4	1·03	[illegible]
[illegible]	[illegible]	Calves	2	10	0·55	[illegible]
[illegible] Lister & Reid [illegible]	Hay and concentrate	Young wethers	1	8	0·22	0·30
[illegible] *et al.* (1961)	Hay and concentrate	Mature wethers	1	8	0·17	0·18
Gibbons (1958)	Hay and concentrate	Identical twin calves	1	2	1·30	1·35
Gibbons (1958)	Hay and concentrate	Identical twin calves	1	6	1·60	1·68
Present work	Hay and concentrate	Lambs	2	6	0·25	0·26

The following observations on the thermodynamics of feeding may partly explain the differences in results from feeding frequency experiments: (*a*) the heat increment of isocaloric rations was unaffected by the frequency at which the rations were given (Blaxter, Graham & Wainman, 1958); (*b*) most of the heat associated with feeding is liberated during the first 6 h after feeding (Brody, 1945); (*c*) Richardson & Mason (1923) demonstrated, with human subjects, that the heat increments of feeds below maintenance level could be utilized in maintaining body temperature when given at intervals of 2 h as no appreciable increase occurred in the basal heat production; (*d*) part of the environmentally induced increase in basal heat production could be compensated for by the heat increment of feeding (Rubner 1902, quoted by Sorensen, 1961).

Several factors can induce increases in the maintenance requirement of animals (Blaxter, 1962). It is possible, therefore, that the differences in results of experiments on frequency of feeding (Table 5) could be an effect of the conditions in which the experiments were conducted and that the effects of frequency of feeding might be most sensitive at or about the maintenance level of feeding. This is substantiated by the experiments referred to in Table 5.

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Haemostatic Failure due to Consumption of Coagulation Factors in Acute Acetic Acid Poisoning[1]

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Local corrosion, intravascular haemolysis, haemorrhagic states, circulatory failure and renal failure are the features of acute poisoning by oral ingestion of concentrated acetic acid (8, 9, 11, 14, 20, 21, 27). The acute renal failure is ascribed to renal damage from circulatory failure and haemolysis (11), but it is difficult to understand the intravascular haemolysis and the bleeding tendency (11).

In the last two years we have treated four patients with acute poisoning from swallowing acetic acid. Signs of haemolysis were evident in all four. In two patients the main features were acute circulatory and renal failure, and one of them required repeated haemodialysis. The haemorrhagic state varied in severity from case to case. Three patients had clinical signs of a mild bleeding tendency, but no definite coagulation abnormalities could be found. This paper is devoted to the fourth case, in which the clinical picture was dominated by a severe bleeding tendency. Coagulation studies showed that it was caused by the consumption of clotting factors.

Case Report

The patient (L. A.), born on 30. 10. 1920, had never been seriously ill before. There was no personal or family history of any bleeding tendency. On the day of admission she drank five glasses of brandy followed by approximately 80 ml. of acetic acid essence, with suicidal intent. Thirty minutes later she was taken to hospital, where immediate treatment was carried out. this comprised approximately

[1] Translated from Dtsch. med. Wschr. 93 (1968), 266.

Tab. I. Laboratory tests on a 48-year-old woman treated for acute poisoning with acetic acid (methods given in brackets, see references)

Test		Time from ingestion of poison								Normal range
		Day of admission				2nd day	3rd day	10th day	16–35 days	
		00.00 hrs.	03.30 hrs.	12.30 hrs.	16.30 hrs.					
Platelets/mm³	(2)	42,500	102,500	—	105,000	110,000	200,000	300,000	230,000	150,000–300,000
Recalcification time (secs)	(6)	> 180	135	120	105	105	105	105	90	90–120
Heparin tolerance test	(16)	> 3.2	—	—	1.4	1.3	1.1	0.9	1.0	0.9–1.1
Prothrombin time (%)	(23)	44	90	—	87	100	100	70	100	70–100
Thrombin time (secs)	(24)	53	27	—	18	13	12	13	13	12–15
Fibrinogen (mg./100 ml.)	(1)	60	145	165	100	210	250	300	260	200–400
Prothrombin %	(26)	57	—	—	—	97	—	—	—	80–100
Factor V %	(17)	56	—	—	—	100	—	—	—	80–100
Factor VII+X %	(18)	65	—	—	—	100	—	—	—	80–100
Euglobulin lysis time (hours)	(19)	> 6	—	—	—	—	—	—	—	> 6
Haemoglobin g./100 ml.		14.6	14.4	—	—	12.3	10.9	9.6	10.5	♀ 14.0 ± 2.0
Leucocytes/mm³		17,300	8600	—	—	14,100	12,600	10,600	9,000	4000–8000
Plasma haemoglobin mg./100 ml.		970	390	—	—	50	—	—		0–0.5
Total bilirubin mg./100 ml.		2.10	2.52	—	—	1.15	0.36	—	0.21	to 1.0
Direct bilirubin		negative	negative	—	—	negative	negative	—	negative	negative
LDH mU./ml.		2100	—	—	—	1050	589	219.6	184	96–241
SGOT mU./ml.		90.4	—	—	—	104.4	18.4	14.9	6.9	5–19
SGPT mU./ml.		40.4	—	—	—	152.5	10.4	13.9	3.6	2.5–17
Alkaline phosphatase units		1.9	—	—	—	—	9.09	—	—	3–15
Neoprotein nitrogen mg./100 ml.		38	—	—	—	—	—	—	—	20–40
Urea nitrogen mg./100 ml.		—	—	—	—	33	22	13.5	11	10–30
Creatinine mg./100 ml.		—	—	—	—	1.35	1.05	1.15	1.2	♀ 0.5–1.1
Sodium mEq./l.		130.4	—	—	—	—	133	142.5	142	135–155
Potassium mEq./l.		4.2	—	—	—	—	3.5	3.85	4.55	3.5–5.5
Chloride mEq./l.		—	—	—	—	—	93	115	95	95–110
E.S.R. mm.		—	5/10	—	—	—	47/81	69/100	44/63	5/12
Urine: protein		—	cloud	—	—	opalescence	—	positive	negative	
bile pigments		—	urobilinogen increased	—	—	normal	—	normal	normal	
red cells		masses	10–20	—	—	2–3	—	0–2	negative	
white cells		—	—	—	—	5	—	0–2	0–2	

3 litres of milk with white of egg, analgetics, antibiotics, calcium, antacids, Vitamin C, and 500 ml. sodium lactate intravenously. The blood pressure—checked at regular intervals—was 130/80 mm. Hg, pulse 80/min., haemoglobin 14.4 g./100 ml., serum haemolysed. She passed 50 ml. of blood-stained urine and vomited increasing amounts of blood (in all, three kidney dishes). After eight hours she was transferred to this hospital.

Findings

An obese 46-year-old woman still in good general condition. Corrosion burns at the angles of the mouth. Oedema of pharyngeal wall and mucosa of hypopharynx, but no obvious corrosion eschars. Epiglottis thickened, arytenoids swollen. Slight dyspnoea, no signs of dehydration. Good circulation in skin and visible mucosal surfaces. No cyanosis. Slight jaundice. No oedema. No lymph-node enlargement. Extensive haematomas at injection sites. Tourniquet test positive. Gagged and vomited blood while being examined. Heart normal. Blood pressure 150/100 mm. Hg, pulse 88. No abnormality on percussion of chest, but there were diffuse rales. Tenderness in epigastrium, no guarding. Liver and spleen not palpable. No abnormality in either renal angle. Genitalia normal on external examination. Skeletal and central nervous systems normal. Chest film and electrocardiogram: no significant abnormality.

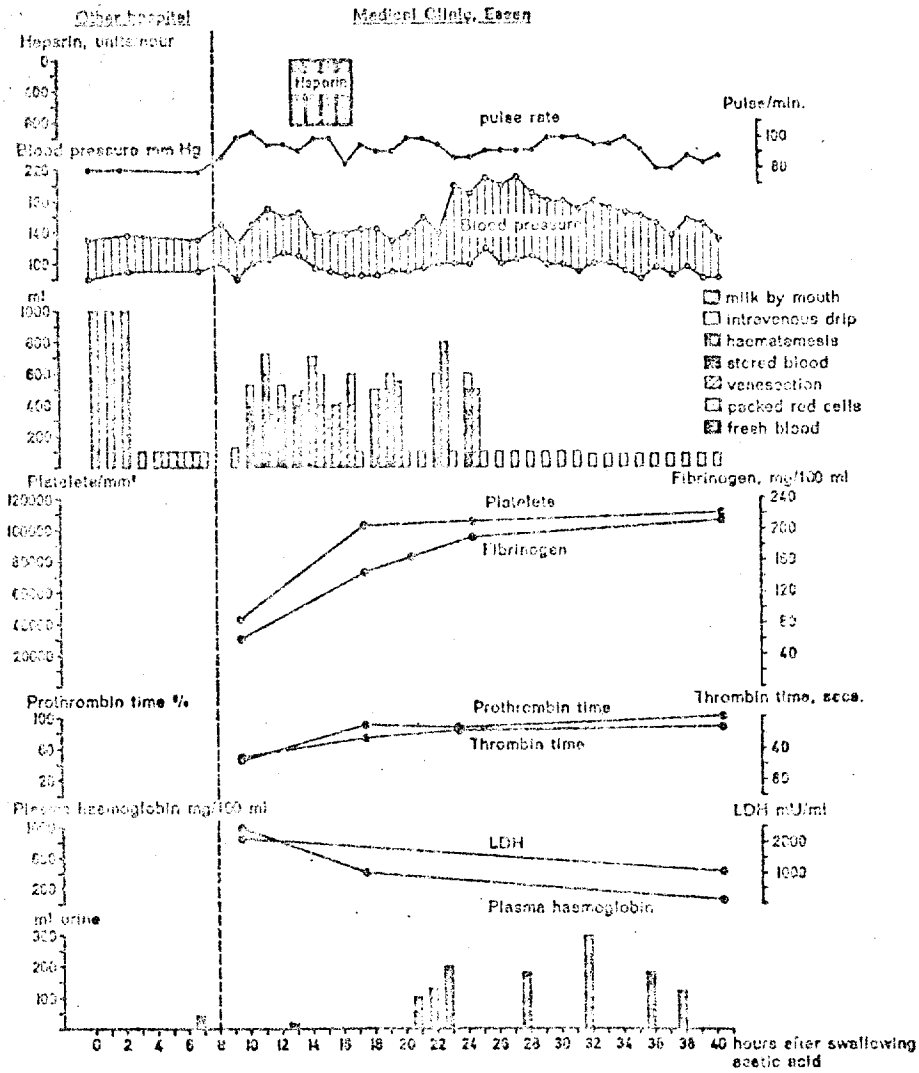

Fig. 1. Pulse rate and blood pressure, platelet count, clotting tests, plasma haemoglobin, lactate dehydrogenase and urinary output in the first 32 hours after swallowing acetic acid. Treatment is also shown.

Haemoglobin 14.6 g./100 ml., white cell count 17,300/mm³, E.S.R. 5/10 mm. (Westergren), serological test for syphilis negative, blood group A Rh, plasma haemoglobin 970 mg./100 ml., serum bilirubin 2.52 mg./100 ml., direct reaction negative, LDH 2,100 mU/ml., SGOT 90.4 mU/ml., SGPT 40.4 mU/ml., nonprotein nitrogen 38 mg./100 ml., serum sodium 130 mEq./l., potassium 4.2 mEq./l. Acid-base balance (Astrup): pH 7.38, PCO_2 32.5 mm. Hg, standard bicarbonate 20.2 mEq./l., base excess —5 mEq./l. Urinary deposit contained masses of red cells.

The clotting studies are set out in Table I.

Course

Two hours after admission her condition began to deteriorate. There was profuse bleeding from the mouth and nose, with haematemesis and passage of blood-stained motions. Laboratory tests showed evidence of intravascular coagulation with concurrent haemolysis (Tab. I). She was given a heparin drip (640 units an hour for four hours) and exchange transfusions. This produced distinct clinical improvement; the clotting tests returned more or less to normal, the signs of haemolysis receded, urinary output increased and the blood pressure temporarily rose (Tab. I, Fig. 1). After 32 hours blood-gas analysis was normal (pH 7.39, PCO_2 44.0 mm. Hg, standard bicarbonate 24.8 mEq./l., base excess + 1.2 mEq./l.). From then on she steadily improved under treatment with infusions, prednisone and antibiotics (Tab. I). Radiological examination 30 days after the episode showed slight inflammatory changes in the lower part of the oesophagus, and hour-glass constriction of the stomach at the level of the angulus with a small residual ulcer on the greater curve. An intravenous pyelogram showed that the left kidney was somewhat smaller than the right, and concentrated the dye less effectively. The serum creatinine (1.2 mg./100 ml.) and clearance studies (inulin clearance 60.0 ml./min./1.73 m², PAH clearance 264.0 ml./min./1.73 m², filtration fraction 22.7%) pointed to some continuing impairment of renal function. There was no further haemorrhage and she was discharged on the 30th day.

Discussion

In the present case acute poisoning by ingestion of concentrated acetic acid caused a haemorrhagic state, intravascular haemolysis, acidosis and renal failure. Among the factors responsible for the bleed-

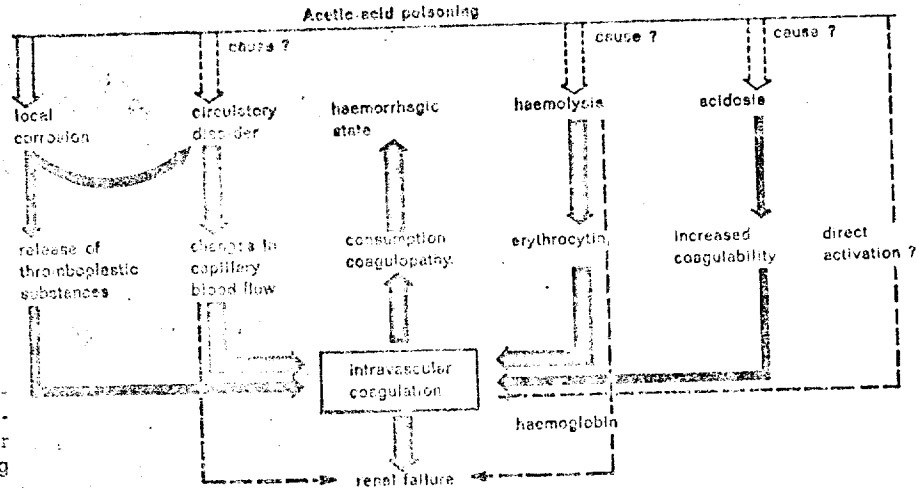

Fig. 2. Diagram of the processes concerned in the pathogenesis of intravascular clotting after acute poisoning by acetic acid.

ing tendency were thrombocytopenia, interference with overall coagulation efficiency (as evidenced by the prolonged recalcification time and reduced heparin tolerance), lengthened prothrombin time and thrombin time, and low levels of fibrinogen. Clotting factor assays showed moderate reduction of prothrombin and Factor V. The activity of the Factor VII complex (Factors VII and X) was still normal. Factor VIII was not assayed, but the serious impairment of overall clotting efficiency in the face of merely moderate prolongation of the prothrombin time indicates that there was in addition some disorder of intrinsic prothrombin activator generation. This combination of thrombocytopenia with coagulation abnormalities consisting of hypofibrinogenaemia and reduction of prothrombin, Factor V and Factor VIII is characteristic of "consumption coagulopathy" (3, 10, 12). Intravascular coagulation consumes platelets, fibrinogen, Factor II (prothrombin), and Factors V and VIII. The resulting haemostatic defect is clinically manifest in a haemorrhagic tendency. The moderate reduction of prothrombin and Factor V indicated that in our patient the condition had not progressed to an advanced stage.

The thrombocytopenia rules out any possibility of primary increase in fibrinolytic activity. Despite the normal euglobulin lysis time, it is possible that there was some reactive fibrinolysis (7), as the initial prolongation of the thrombin time, like the hypofibrinogenaemia, could have been caused by breakdown products of fibrinolysis (Antithrombin VI). As shown in Fig. 2, the possible causes of intravascular clotting which must be considered in the present case are haemolysis, circulatory abnormalities, local corrosive damage and acidosis. Direct activation of the clotting system by concentrated acetic acid is another possibility to be taken into account.

Intravascular coagulation as an isolated phenomenon following the ingestion of concentrated acetic acid has not previously been reported, but intravascular coagulation associated with acute haemolysis is well known (3, 10, 12, 22). However, only when accompanied by a circulatory disorder does the release of thromboplastic material (erythrocytin) from erythrocytes (23) cause intravascular clotting (4). In the present case there were no clinical signs of shock, though no information is available for the first 30 minutes after taking the poison. The absence of a generalized circulatory disorder does not rule out the possibility of local abnormalities (3) and in fact the acute renal failure strongly suggests that they occurred. It is also likely that the extensive local corrosive lesions of the upper alimentary tract caused serious microcirculatory abnormalities. Changes in flow conditions in the capillaries are an important factor in causing intravascular clotting (3, 12). It is also possible that the clotting system was activated by thromboplastic substances released from the corrosive lesions or as a result of the acidosis (5). There is still uncertainty regarding the pathogenesis of the haemolysis and acidosis which follow ingestion of concentrated acetic acid.

In treating haemostatic failure due to consumption of clotting factors, it is advisable to give heparin so as to interrupt the process of intravascular clotting (7, 10, 12, 13, 15, 28). In the present case continuous infusion of heparin for four hours restored virtually normal coagulation function and also effected a brisk diuresis. Simultaneous treatment of the haemolysis by exchange transfusion rapidly brought down the high levels of plasma haemoglobin and serum lactate dehydrogenase.

Summary

A 46-year-old woman took 80 ml. of concentrated acetic acid by mouth. The ensuing haemorrhagic state proved to be due to consumption of clotting factors as a result of intravascular coagulation. Among the factors responsible for the latter are intravascular haemolysis, circulatory disorders, local corrosive

lesions and acidosis. Treatment by exchange transfusion and a continuous heparin drip restored normal clotting function and urinary output.

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Reports of Cases.

TWO FATAL CASES OF POISONING BY ACETIC ACID.

By Arthur A. Palmer, M.B., F.R.C.S. (Edinburgh),
Government Medical Officer, Sydney.

Deaths from the taking of acetic acid are, I think, very rare in this country, in Great Britain and the United States of America. They are said to occur quite frequently in some countries, especially in Russia. I know of only two cases, and short notes of them might be of interest.

An old man, aged seventy-six, was seen about midnight to drink something from a bottle, and a little later staggered across the floor and fell. The one-ounce bottle was labelled "glacial acetic acid", of which it smelt strongly, and the patient admitted he had swallowed the whole bottle full in an attempt to commit suicide. He died about three hours later in the Parramatta District Hospital and the clinical notes were kindly supplied to me by Dr. A. C. Saunders.

When seen at 12.35 a.m. the patient was in a condition of shock, with a temperature of 36.3° C. (97.4° F.), a pulse rate of 128 and a respiration rate of 30 per minute. His breath smelt strongly of acetic acid. His lips and tongue were burnt and he complained of burning from his mouth down to his stomach. He perspired freely and had a cold skin throughout. There was no diarrhœa.

Owing to the length of time from the taking of the acid and consequent risk of using a stomach tube, the stomach was not washed out, but the patient was given lime water to drink and later soapy water. This was followed by vomiting, the vomitus smelling of acetic acid. Further drinks of soapy water and of milk were given, and at 1 a.m. some of the vomitus was blood stained. At 12.45 a.m. 2·2 milligrammes (one-thirtieth of a grain) of strychnine were given, and at 1.10 a.m. 0·016 gramme (one-quarter of a grain) of morphine. The patient's condition was now moderately good, with slow and deep respiration and a good pulse until 3.20 a.m., when the pulse became soft and rapid, and the patient died at 3.30 a.m. No autopsy was made, but the *post mortem* appearances can be taken from the following.

A woman, aged thirty-three years, died after drinking an unknown quantity of acetic acid, and an autopsy was carried out at the Sydney morgue.

The lips showed burning. The stomach contents smelt strongly of acetic acid and its mucous membrane was deeply inflamed, in parts showing a rich crimson colour. The upper part of the small intestines was affected similarly, but to a less degree. The blood was very dark and the lungs, liver, spleen and kidneys were engorged with dark blood.

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THE UPTAKE AND EXCRETION OF WATER IN RATS POISONED WITH LEAD

BY

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The main injury produced by lead in rats' kidneys affects particularly the loop of Henle and the distal tubules (Pardoe, 1952). As there are grounds for associating these regions with the site of antidiuretic action of postpituitary extracts (cf. Pitts and Sartorius, 1950, for discussion), the excretion of water and the effect of vasopressin have been examined in rats poisoned with lead. Observations have been made also on the absorption of water from the alimentary canal and on its distribution in the body. This aspect of lead poisoning does not appear to have been investigated systematically previously, apart from a paper by Masciotta (1942), to which we have been unable to obtain access.

METHODS

Male albino rats from the local colony were used throughout. Some observations were made on the chronically poisoned rats described in the previous paper (Pardoe, 1952), and this set of rats will be referred to here as series I. Another chronic series (II) and two acute series (III and IV) of experiments were performed. Details of the doses used are shown in Table I. The rats weighed initially about 250 g. (series I and II) or 200 g.

TABLE I

DETAILS OF DOSAGE OF RATS WITH LEAD ACETATE OR SODIUM ACETATE

Exp. series No.	Cation	Single dose M-eq./kg.	No. of doses	Duration of dosing days	Route of administration	Comment
I[1]	Pb	3.3	27 followed by	63	Oral	Dosing three times weekly throughout
I[2]	Na	13.3	31	72		
II[1]	Pb	13.3	29	98	Oral	Dosing three times weekly for first 18 doses, next three doses in four weeks, then twice weekly
II[2]	Na					
III[1]	Pb	13.3	2	7	Oral	
III[2]	Pb	0.67	1	—	i.v.	
III[3]	Pb	6.7	2	7	Oral	Intravenous dose given at same time as first oral dose
		0.33	1	—	i.v.	
III[4]	Na	6.7	2	7	Oral	
		0.33	1	—	i.v.	
III[5]	—		—	—	—	No treatment
IV[1]	Pb	0.67	1	—	i.v.	
IV[2]	Na					

(series III and IV) with standard deviations of about 10 per cent. The doses used generally were near to those expected to cause death, and dosing was stopped at certain times because the rats were weak or lethargic. The doses given intravenously were injected over periods of 40–60 sec. for each rat. Immediate fatalities were usual if this rate was exceeded, but no deaths occurred at the rate adopted. In all the experiments described no deaths occurred which could be attributed to the quantity of lead given to the animals.

Water absorption experiments.—The method of Heller and Smirk (1932) was followed. Rats were given lukewarm tap water (5 ml. per 100 g. body weight) by stomach tube, and were killed in groups of three at various times afterwards. Some rats were killed without giving them any water. The alimentary canal, from the lower end of the oesophagus to the rectum, was removed, with care not to lose any of its contents, and was weighed at once. All weights were expressed per 100 g. body weight and the mean amount of water absorbed was calculated from the mean weight of the stomach and intestines of the unhydrated animals plus five minus the mean weight of the stomach and intestines of the hydrated animals at a given time.

Tissue water content.—The water content of tissues was determined from their fresh weight and their weight after heating to 110° C. for eighteen to twenty-four hours in waten glasses or platinum crucibles.

Water diuresis experiments.—Food was withheld overnight, but access to water was allowed. In the morning each rat was given lukewarm tap water (5 ml. per 100 g. body weight) by stomach tube and placed either in a metabolism cage constructed on a glass funnel of 15 cm. maximum diameter or in a group of six rats in larger galvanized metal cages. Vasopressin ("Pitressin," Parke Davis: 0.001 or 0.0005 unit per 100 g. body weight) or nicotine hydrogen tartrate (0.25 mg. of nicotine base per 100 g. body weight) was injected subcutaneously immediately after the water if either of these drugs were being studied. The total urinary output was observed approximately every quarter of an hour until the rate of excretion had risen and become low again. The results were plotted graphically, and as a rule the time until 50 per cent of the dose of water had been excreted was determined from these graphs. The amount excreted at different times was similarly obtained graphically, and mean values were obtained from replicate estimates. The passing of a small volume of urine shortly after hydration and before the onset of diuresis, as described by Burn (1931), did not appear to us sufficiently important to render the use of the 50 per cent time unreliable. In some experiments an unusually gradual diuresis occurred in which there was no definite peak in the rate of excretion: the time to the peak rate was therefore not a feasible measure of the delay in diuresis for our purposes.

Inactivation of vasopressin by liver.—The method used was based on that described by Birnie, Blackmore, and Heller (1952). A normal or a lead-poisoned rat was killed and bled from the carotid artery and its liver was removed; 1 g. of liver was transferred to an ice-cold homogenizer and ground for 5 minutes with 2 ml. of ice-cold water. 3 ml. ice-cold water were added to the homogenate and ground further, after which the material was made up to 10 ml. with distilled water; 1 ml. aliquots were added to a series of centrifuge tubes. To these centrifuge tubes were then added 3 ml. of ice-cold 0.15 phosphate buffer of *p*H 6.5 and 1 ml. vasopressin in different concentrations. The tubes were shaken and incubated at 37° C. for 30 minutes, and the presence or absence of antidiuretic activity in their contents was then demonstrated by injecting 0.5 ml. of the incubated solution intraperitoneally into each of four rats, which had just been hydrated with 5 ml. water per 100 g. by stomach tube, and observing their rate of urinary secretion.

Action of vasopressin on the weight of frogs.—The effect of vasopressin (0.5 unit per 20 g. frog) on the weight of frogs was determined as described by Heller (1942). Some of the frogs tested were previously exposed to lead by keeping them for two to three weeks

in water which contained 1 mM. lead chloride per litre and which was changed three times a week. One frog out of twenty-four died under these conditions, compared with none out of twelve which were kept in distilled water in an adjacent tank.

Error of experimental results.—In the experiments on the absorption of water, on the quantity of water in tissues, and on the effect of vasopressin on the weight of frogs, the variances of the different groups in a single experiment appeared to be independent of the means of the groups. Pooled estimates of the variance due to experimental error were therefore made by the usual methods of the analysis of variance, and were used to test the significance of observed differences when the analysis showed that the effects of treatment were larger than those attributable to chance variation. In the diuresis experiments the variance between replicate observations was clearly related to the value of the mean, although the relation was not a simple one. The variance and standard error were therefore calculated separately for each mean.

RESULTS

Absorption of water from the alimentary canal

The absorption of water from the alimentary canal was studied in the rats of series II and III. The results with series III are shown in Fig. 1. Essentially

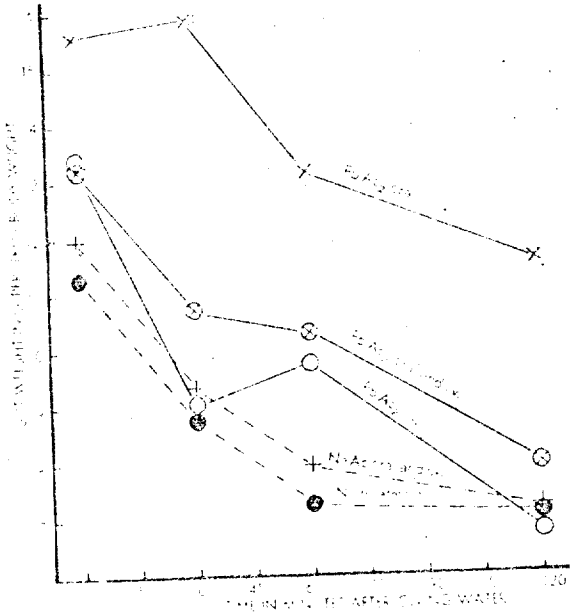

FIG. 1.—The absorption of water from the alimentary canal. Ordinates: mean weight of the alimentary canal and contents in g. per 100 g. body weight for groups of three rats. Abscissae: time in minutes after giving water, 5 ml. per 100 g. body weight, by stomach tube. ×——× After lead acetate orally. ⊗——⊗ After lead acetate orally and intravenously. ——, After lead acetate intravenously. - - - - + After sodium acetate orally and intravenously. ● - - - ● No previous treatment. Details of treatment are given in Table I. The values at 0 minutes were obtained by adding 5 g. per 100 g. to the mean weight of the alimentary canal when no water was given. The standard error of each of the points, estimated by the analysis of variance, is ±0.53 g. per 100 g. body weight.

similar results were obtained with the chronically orally poisoned rats of series II. In both series, the alimentary canals of the rats which had received lead were considerably heavier than those of the control rats. This occurred particularly when lead acetate had been given by stomach tube, but it was definite also when it had been given entirely intravenously. The increased weight was partly due to an increase in the weight of the actual tissues and partly to an increase in the contents (Table II). The increase in contents was particularly prominent when the lead had

TABLE II

THE WEIGHTS OF VARIOUS ORGANS IN RATS AFTER ADMINISTRATION OF LEAD ACETATE OR SODIUM ACETATE

Details of dosage are given in Table I. The rats of series III were killed eight days after beginning dosing and the rats of series II were killed 100–104 days after beginning dosing. All values are the means for groups of three rats, except that the values for series II^1 and II^2 normals are for groups of two rats. Standard errors of the means in each group have been obtained from the analysis of variance

Exp. series No.	Mode of dosing	Organ weights, g. 100 g. body weight						Water content % of organ wt.	
		Stomach	Small intestine	Gut contents	Adrenals	Kidney	Liver	Kidney	Liver
III^1	Acute oral $PbAc_2$	0.64	3.07	6.0	0.021	0.89	3.21	79.7	70.2
III^2	Acute i.v. $PbAc_2$	0.62	3.32	3.4	0.025	1.08	4.57	80.1	72.3
III^3	Acute oral and i.v. $PbAc_2$	0.65	3.26	3.8	0.022	1.00	3.89	79.2	71.8
III^4	Acute oral and i.v. NaAc	0.51	2.41	3.0	0.018	0.75	3.08	78.4	70.1
III^5	No treatment	0.53	2.75	2.6	0.021	0.73	3.12	77.8	70.5
	Standard errors of series III means	±0.03	±0.17	—	±0.001	±0.06	±0.21	±0.45	±0.25
II^1	Chronic oral $PbAc_2$	0.82	2.60	—	0.015	1.24	3.52	81.1	70.1
II^1	Chronic oral $PbAc_2$, water withheld for 24 hr. before death	0.80	2.38	—	0.021	1.13	3.91	75.0	70.3
II^2	Chronic oral NaAc	0.51	1.45	—	0.014	0.70	—	76.5	—
II^2	Chronic oral NaAc, water withheld for 24 hr. before death	0.57	2.02	—	0.015	0.65	2.38	75.9	68.8

been given by stomach tube. The control rats, which had received sodium acetate, had slightly more material in their alimentary canals both before and after water was given than had the rats which had had no previous treatment. The differences were trivial compared with those observed after lead, and they might have been due to chance ($t=1.33$, $P \simeq 0.2$). After water had been administered, the rate of absorption in the controls followed the expected course (Fig. 1). In the rats which had received lead acetate orally, the onset of absorption was delayed. Indeed, the mean weight of the gastro-intestinal tract half an hour after the administration of water was greater than could be accounted for by the original weight of the tract plus the administered water, both in series III (Fig. 1) and series II. The excess in series III could be due to the chances of sampling ($t=1.47$, $P \simeq 0.2$), and the weights of the alimentary canals of unhydrated rats in series II were obtained on a different day from those of the hydrated rats and so were not strictly comparable. The evidence is therefore only suggestive that there was an actual influx of material to the gastro-intestinal tract in these animals, but there is no doubt that absorption was delayed. Apart from this delay or influx, absorption proceeded normally or, in the rats which received lead acetate intravenously, if anything unusually quickly; though the results from these last rats were rather irregular.

The effect of nicotine hydrogen tartrate (0.25 mg. of nicotine base per 100 g. body weight) on the absorption of water was observed in some normal rats, in some

rats chronically poisoned with lead acetate by stomach tube, and in some acutely poisoned intravenously. Nicotine had no significant effect on the weight of the gastro-intestinal tract when no water was given. It tended to retard the absorption of water. For example, in the rats of series II, which had received sodium acetate, the mean weight one hour after giving water was 8.1 g. per 100 g. body weight when no nicotine was given and 10.1 g. per 100 g. when nicotine was given at the same time as the water ($t = 2.32$, $0.1 > P > 0.05$). Similar differences were seen in the lead-poisoned rats of series II (13.9 and 16.4 g. per 100 g. body weight without and with nicotine; $t = 2.90$, $0.05 > P > 0.02$). As these differences are in the same direction, they are very unlikely to be due entirely to chance. This point was not examined in more detail because the slowing of absorption was not very large and did not appear to be an important factor in the observed changes in the action of nicotine on water diuresis.

Organ weights and water content of tissues

As well as the stomach and small intestine, the liver, kidneys, and adrenals were always heavier in the rats which had received lead acetate (Table II). The greater weight of the livers and kidneys was due mainly to an increase in dry weight but partly also to an increase in water content. The increase in dry weight occurred surprisingly quickly. In a pilot experiment, 18 hours after lead or sodium acetate had been given by various routes, the dried livers of the animals to which lead had been given were on the average 21 per cent heavier and the dried kidneys 19 per cent heavier than those of the controls. At this time differences in water content were not significant. By eight days slight increases in the tissue water content were found consistently in the liver and kidney, though not definitely in the stomach and small intestine.

Water balance

When groups of three rats were placed in metabolism cages with or without access to water for twenty-four hours, the weight losses, urinary output, and fluid intake of the control rats and of the rats which had been receiving lead acetate for the previous three months did not differ greatly. However, the leaded rats, which were not allowed access to water, passed more urine than the controls (4.2 instead of 2.6 ml. per 100 g. body weight) and lost more weight (10.9 instead of 8.9 per cent) and had larger adrenals (Table II). The variation between the urine output of individual rats was not measured and the observations need to be repeated with more animals.

Excretion of water by the kidneys

When water was given to lead-treated rats, fairly normal diuresis occurred. Immediately after giving lead, the responses were irregular, possibly because the rats were excited after their recent handling (Table III, rats of series III). Later, the observed 50 per cent times were within normal limits (cf. Dicker, 1951, for references), except, rather surprisingly, for the control rats of series II. The rapidity of diuresis decreased in these rats after sodium acetate was administered, and they then always took over 120 minutes to excrete half their dose of water. This tardiness was not observed in the rats of series I, which also received sodium acetate, and it has no obvious explanation. It makes the much faster excretion by the lead-

treated rats of doubtful importance, especially as this faster rate was within the normal range and as the lead-treated rats of series I excreted water not significantly faster than their controls (t 1.13, 0.3 P 0.2). Possibly lead causes some acceleration of normal diuresis, but the effects are not striking.

Effect of vasopressin on the excretion of water.—The results of experiments in which 0.004 unit of vasopressin ("Pitressin," Parke Davis) per 100 g. body weight was injected subcutaneously at the same time as water (5 ml. per 100 g.) was given by stomach tube are also shown in Table III and Fig. 4. In acutely poisoned rats (series III) the three groups which had received lead all started to show a diuresis sooner than the controls and excreted half the dose more quickly, but the difference between the control and treated groups was not larger than differences commonly observed between identically treated groups on the same day. In rats which were receiving repeated doses of lead or sodium acetate by stomach tube (series II) the mean delay in the sodium group was reasonably stable. In the lead group there was an initial period in which the sensitivity to vasopressin was much increased, after which the animals became abnormally insensitive. In these rats the hypersensitivity reappeared after a period between the sixth and ninth weeks in which

TABLE III

WATER DIURESIS IN RATS TREATED WITH LEAD ACETATE OR SODIUM ACETATE, AND THE EFFECT OF VASOPRESSIN AND NICOTINE

The figures given are the time in minutes from the administration of water (5 ml. per 100 g. body weight) by stomach tube to the time at which a volume of urine equal to half this dose had been passed. Details of dosage with lead acetate or sodium acetate are given in Table I. Vasopressin or nicotine was injected subcutaneously immediately after the water was administered. The results in series I were obtained from nine rats observed individually, in series II from eighteen rats observed in groups of six, in series III from six rats observed in a single group, and in series IV from five rats observed in a single group.

Exp. series No.	Method of treatment	No. of doses given	Days from last dose	Time in minutes to half excretion: Plain water diuresis Pb	Plain water diuresis Na	Vasopressin 0.004 u. 100 g. Pb	Vasopressin 0.004 u. 100 g. Na	Nicotine 0.25 mg. 100 g. Pb	Nicotine 0.25 mg. 100 g. Na
III[1]	Oral	1	1	136	—	254	—	—	—
III[2]	Intravenous	1	1	136	—	230	—	—	—
III[3,4]	Oral and intravenous	1	1	96	95	250	310	—	—
III[5]	No treatment	—	—	—	97	—	284	—	—
IV	Intravenous	1	1	—	—	—	—	163	97
II	Oral, large doses	0	—	107		—	—	—	—
		10–13	1	82	—	254	196	—	—
		15–18	1	82	141	114	183	246	130
		19	3	79*	134*	—	—	—	—
	Oral, reduced doses	20	3	80	125	—	—	—	—
		20	8	97*	139*	—	—	141*	141*
	Oral, large doses	26–28	2	72	124	216	202	677	167
I	Oral (± S.E.)	58	1–2	75 ± 3.8	82 ± 5.4	94 ± 8.8	133 ± 9.1	—	—
	Oral (± S.E.)	58	75–83	—	—	104 ± 6.7	135 ± 4.6	—	—

* Observations on one group of six rats.

the dosage of lead had been greatly reduced (Fig. 2) and the insensitivity did not reappear before they were killed. In the other long series (I), diuretic responses were not studied at all until lead acetate had been administered for eighteen weeks continuously. At this time the rats which had received lead were notably less sensitive to vasopressin than were the controls (Fig. 2): and in these rats the insensitivity persisted for at least ten weeks after the administration of lead acetate had been stopped. In these animals at the end of eighteen weeks' treatment, the difference in the mean time to excrete half the dose of water after vasopressin was highly

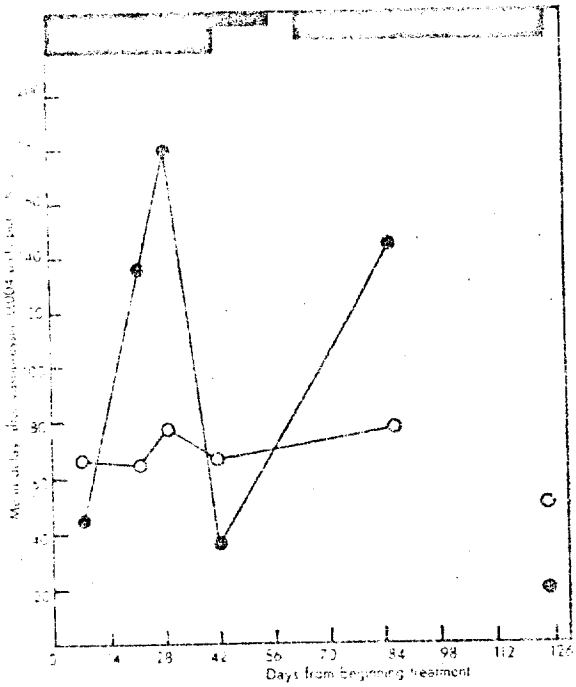

FIG. 2.—The effect of vasopressin at various times during the administration of lead acetate. Ordinates: Delay in time to fifty per cent excretion of dose of water given by stomach tube when vasopressin (0.004 unit per 100 g.) was given subcutaneously immediately after the water. Abscissae: Time in days from starting administration of lead acetate. ●——● Rats treated with lead acetate. ○——○ Rats treated with sodium acetate. The points joined by lines are for rats of series II. The isolated points at 124 days are for rats of series I. The blocked figure at the top of the graph represents the dose of lead acetate per week and applies to series II: dosage in series I was of the same order of magnitude (see Table I), but did not follow the detailed course indicated.

significant ($P < 0.01$). As the rats to which lead had been given excreted water slightly more quickly than the controls, the difference between the delays produced by 0.004 unit vasopressin per 100 g. is less than the difference between the times to half excretion, but it is still substantial (18.6 instead of 50.5 minutes) and also highly significant ($P < 0.01$). In terms of the dose-response curve obtained in this laboratory for the delay of diuresis by vasopressin in normal rats, this difference indicates that 0.004 unit of vasopressin per 100 g. body weight was having about as much effect in the leaded rats as 0.0014 unit of vasopressin per 100 g. body weight would be expected to have in the controls.

Effect of nicotine on the excretion of water.—The effect of nicotine hydrogen tartrate (0.25 mg. of nicotine base per 100 g. body weight) injected subcutaneously immediately after water had been given was examined in the rats of series II and IV. It caused a much greater delay in those rats which had been exposed to high doses of lead immediately previously than in control rats, or in rats which had not

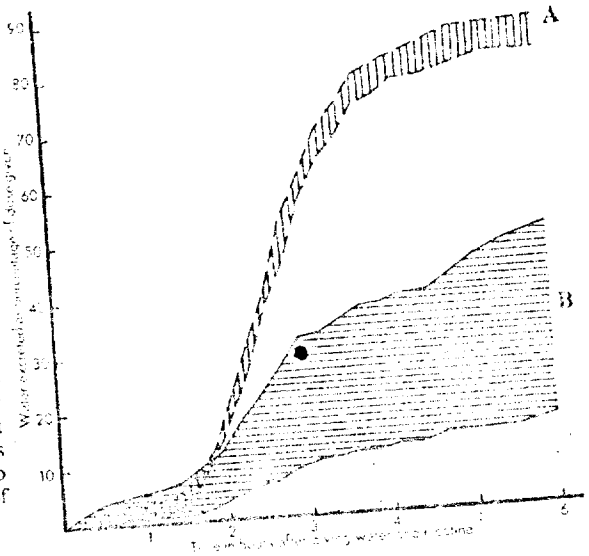

FIG. 3.—Water diuresis after nicotine in lead-poisoned and control animals. Ordinates: Percentage of dose of water (5 ml. per 100 g. body weight) excreted by groups of six rats. Abscissae: Time in minutes from administration of water by stomach tube. A, range of three groups of six control rats. B, range of three groups of six lead-poisoned rats. Nicotine hydrogen tartrate (0.25 mg. nicotine base per 100 g. body weight) was injected subcutaneously immediately after water was given. This experiment was performed on rats of series II 85 days after beginning to give lead acetate. Details of dosage are given in Table I.

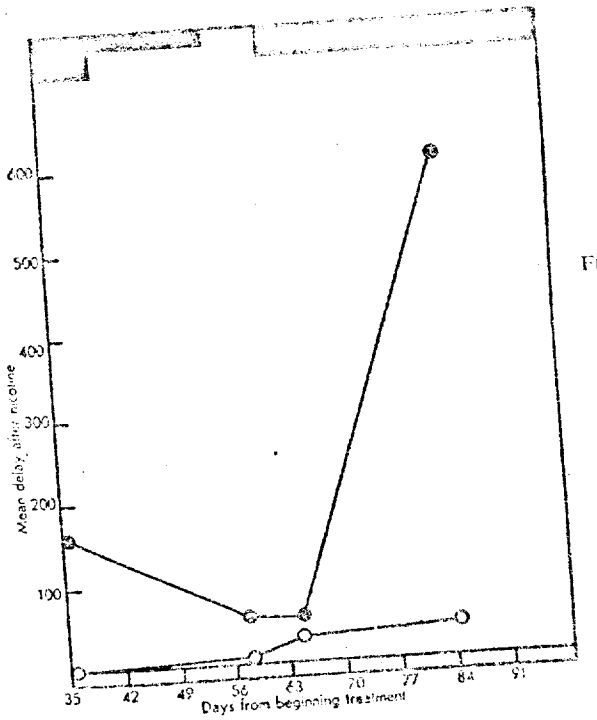

FIG. 4.—The effect of nicotine at various times during the administration of lead acetate. Ordinates, abscissae, and symbols, but not scales, as in Fig. 2. All the points are for rats of series II.

received lead for some days (Table III and Figs. 3 and 4). The dose of nicotine used had much less effect than 0.004 unit of vasopressin per 100 g. in the control animals but much more effect in animals which had just received large doses of lead, and in the latter the effect of nicotine persisted for many hours (Fig. 4). Nicotine if anything delayed the absorption of water. However, nicotine exerted the same very prolonged antidiuretic action when it was injected fifty to sixty minutes after water had been given, at a time when at least three-quarters of the water had been absorbed and only one-quarter excreted. The prolongation is therefore not substantially dependent on changes in water absorption, though these may contribute to it. There was no obvious difference in the frequency of convulsions or the degree of prostration produced in the two groups of rats, so the lead-poisoned rats did not appear to be generally hypersensitive to nicotine.

Destruction of vasopressin by the liver.—Various quantities of vasopressin were incubated for thirty minutes at 37° C. with homogenates of liver taken from normal and from lead-poisoned rats of series II, and their antidiuretic activity was then compared. It was found that 1.0 g. of normal rat liver under these conditions

TABLE IV

INACTIVATION OF VASOPRESSIN ON INCUBATION WITH HOMOGENIZED LIVER

Exp.	Concentration of vasopressin in incubated suspension of liver	Dose of suspension injected into rats	Time of excretion of half of dose of water: Liver from normal rats	Time of excretion of half of dose of water: Liver from rats poisoned with lead
	unit/ml.	ml./100 g.	min.	min.
1	0.00	0.3	113	115
	0.002	0.3	100	136
	0.002*	0.3	145*	155*
2	0.00	0.3	91	106
	0.02	0.3	120	102
	0.16	0.3	163	147

* Vasopressin added after incubation.

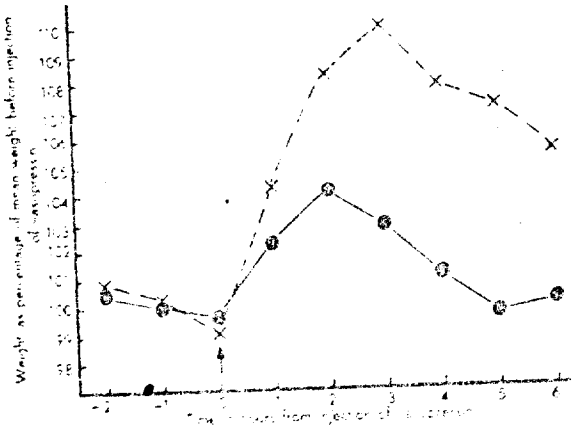

FIG. 5.—The effect of vasopressin on the weight of normal and lead-poisoned frogs. Ordinates: Mean weight of a group of four frogs, as a percentage of the mean weight in three successive hourly observations. Abscissae: Time in hours from injection of vasopressin (0.5 unit per 20 g.). ---- Normal frogs. ● ----- ● Frogs kept in lead chloride (1 mM. litre) for 21 days before experiment.

completely inactivated 0.27 unit of vasopressin, and partially inactivated 0.81 unit. As Table IV shows, there was no appreciable difference in the antidiuretic activity of vasopressin which had been incubated with normal liver and that which had been incubated with the same weight of liver from rats poisoned with lead.

Effect of vasopressin on the weight of frogs.—The effect of vasopressin in doses of 0.5 unit per 20 g. on the weight of normal and lead-poisoned frogs was observed in two sets of experiments, each on four pairs of frogs. The mean results of one experiment are shown in Fig. 5. Vasopressin caused a much larger increase in the weight of the normal frogs than the lead-poisoned frogs, and an analysis of variance showed that in each experiment the difference would have occurred by chance less than once in a thousand experiments.

Discussion

The primary object of these experiments was to demonstrate whether the histologically demonstrable injury produced by lead salts in the renal tubules was associated with a reduced sensitivity to posterior pituitary extracts. The evidence on the whole suggested that this was so, but it was clear that other changes in the absorption and excretion of water took place in lead poisoning and complicated any interpretation of the observations. Most of these changes need to be investigated further, and our object is mainly to draw attention to their occurrence.

The alimentary canals of rats to which lead had been administered were dilated and contained more material than usual. This was not surprising when large doses of concentrated solutions of lead salts had been given by stomach tube, but it was observed also after intravenous administration when direct astringent and osmotic effects on the mucosa were avoided. Lead appears in the stomach wall and in its contents after intravenous injection of lead salts in rabbits (Ginsburg and Weatherall, 1948), and it is reasonable to expect a similar finding in rats; nevertheless the quantity of lead so reaching the contents of the alimentary canal in our rats would be unlikely to be more than about one-thousandth of the dose given by stomach tube. After dosing by stomach tube, absorption of water was delayed; after intravenous injection of lead there appeared to be no slowing of absorption and so the increase in the contents is more likely to be due either to stimulation of secretion or to interference with the musculature of the alimentary canal than to primary failure of absorption of material taken by mouth. As the effect was seen after intravenous injection of doses much smaller than those used by stomach tube, it is more probably due to a central or reflex action of lead than to a purely local action in the alimentary canal itself.

In addition to the increased contents, the stomach and intestines themselves were heavier in the animals to which lead had been given, and this was true also of the liver and kidneys. This increase was due partly to an increase in dry matter and partly to an increase in water content. We have no evidence about how the increments in dry weight occurred. The increase in water content was not great. For example, in the rats of series III the mean water content of the kidneys for all the control animals was 78.1 per cent and for all the leaded animals was 79.7 per cent. The corresponding figures for the liver were 70.3 and 71.4 per cent. However, during water diuresis, the water load of the tissues, calculated as the difference between the water absorbed from the gut and the water excreted by the kidneys,

is of the order of 2 per cent of the body weight. This water is probably not distributed uniformly, and we have not found comparable differences, for example, in skeletal muscle. However, differences of 1.1 to 1.6 per cent in the water content of some tissues before hydration may well affect the response of the body to water. From preliminary observations not reported here it appears that the water content of at least the kidneys in leaded animals rises less than in normal animals during water diuresis: and this appears to be of considerable importance in interpreting the results.

The part played by the adrenals also requires investigation, since they have commonly been enlarged in lead-poisoned animals and it is at least possible that this enlargement is associated with changes in functional activity.

Differences in the diuretic responses of normal and lead-treated rats have, therefore, to be considered against a background of differences in initial tissue hydration and possibly in the rate of water absorption. Plain water diuresis was not greatly altered. There was perhaps a little acceleration, but the evidence, discussed above, is equivocal. The antidiuretic action of vasopressin was at first enhanced and later diminished. A considerable reduction in sensitivity to vasopressin was seen also in frogs poisoned with lead. The inhibition of water diuresis by nicotine was much augmented four to six weeks after exposure to lead had begun, and it appeared that this augmentation depended upon the maintenance of a high dosage of lead. It disappeared when dosing was reduced and reappeared when it was increased again. The responses to nicotine were clearly distinct from those to vasopressin at the same time.

The insensitivity to vasopressin developed at about the same time as histological changes appeared in the renal tubules (Pardoe, 1952), and was presumably due to the injury to those cells. There was no visible evidence of earlier injury and it is difficult to account for the hypersensitivity to vasopressin observed about the fourth to sixth weeks of poisoning. Possibly lead initially increased the sensitivity of the tubular cells before visibly damaging and desensitizing them. The tubular injury involved both the loop of Henle and at least the distal convoluted tubules, but exact identification of the affected parts of the tubules was difficult in the presence of the structural damage caused by lead. The present observations do not, therefore, provide much useful additional evidence about the site of the antidiuretic action of posterior pituitary extracts.

The hypersensitivity to the antidiuretic action of nicotine has to be accounted for separately, since it was conspicuous in animals which responded more or less normally to vasopressin, and in animals which were relatively insensitive to vasopressin. Some evidence was obtained of delayed absorption of water under the influence of nicotine both in normal and in lead-poisoned animals, but the characteristic prolonged reduction in the rate of urine flow was produced in lead-poisoned animals when the nicotine was injected an hour after giving water, at a time when absorption was nearly complete. Unless nicotine has antidiuretic actions by some other pathway than stimulating the pituitary-hypothalamic mechanism, it is simplest to assume that this mechanism is rendered more sensitive by lead, so that a single stimulus has a much larger or more prolonged effect than normal. We have already argued that the observed effects of lead given orally and intravenously in producing dilatation of the stomach are probably of central origin, and it seems

plausible that both these changes indicate actions of lead on vegetative centres in the central nervous system. However, this is only a speculative interpretation and further experiments are needed to support or refute it.

SUMMARY

1. Some functions of the kidneys and the amount of water in certain organs under various conditions have been studied in rats poisoned with lead acetate. Rats which had been treated with equivalent amounts of sodium acetate were used as controls.

2. The onset of absorption of water from the alimentary canal was delayed in rats which had received lead acetate by stomach tube, but then proceeded normally. It was little affected when lead had been given intravenously. Absorption was possibly also somewhat delayed both in normal and in leaded rats by nicotine.

3. In the rats which had received lead acetate, the stomach, small intestine, liver, kidneys, and adrenals were heavier than in the controls. In at least the liver and kidneys there was both an increase in the dry weight and an increase in the water content of the organs. There was also an increase in the contents of the alimentary canal, particularly but not only in the rats which had received lead by stomach tube.

4. Water diuresis was slightly but not significantly accelerated in rats to which lead acetate had been given repeatedly by stomach tube.

5. The inhibitory effect of vasopressin on water diuresis in rats poisoned with lead was variable. In the first few weeks of administering lead, vasopressin had more effect than in normal rats. Later the animals became abnormally insensitive, at first reversibly and later apparently irreversibly.

6. In rats which were receiving large doses of lead acetate frequently, the anti-diuretic effect of nicotine was greatly increased. This increase was not related to an increased sensitivity to vasopressin. It disappeared within a few days of reducing the dose of lead and reappeared when intensive dosing was resumed.

7. Vasopressin in very large doses had highly significantly more effect on the weight of normal frogs than it had on the weight of frogs which had been kept in 0.001 M lead chloride in the previous two to three weeks.

8. Possible causes of these changes are discussed.

We are much indebted to Professor H. Heller for giving us details of the method of measuring the inactivation of vasopressin by the liver, and to Miss H. Moore for technical assistance.

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Total Synthesis of Acetate from Carbon Dioxide. Retention of Deuterium during Carboxylation of Trideuteriomethyltetrahydrofolate or Trideuteriomethylcobalamin†

Donald J. Parker,‡ Harland G. Wood,* R. K. Ghambeer,§ and Lars G. Ljungdahl

ABSTRACT: A mass analysis of acetate formed from trideuteriomethylcobalamin or 5-trideuteriomethyltetrahydrofolate in fermentations of α-ketobutyrate by an enzyme preparation from *Clostridium thermoaceticum* has shown that mono-, di-, and trideuterioacetate are formed. More than 50% was trideuterioacetate whether the precursor was trideuteriomethylcobalamin, 5-trideuteriomethyltetrahydrofolate, or a 1:1 mixture of the deuterio and hydrogen methyl precursors. No deuterium isotope effect was observed in the formation of acetate. Three mechanisms have been considered: (1) a Grignard-type reaction involving addition of CO_2 to a methyl carbanion, (2) carboxylation of the methyl group with displacement of hydrogen to form a carboxymethyl group and then return of the displaced hydrogen to form the acetate by an intramolecular reaction, and (3) as in two but the reaction to form acetate is by an intermolecular reaction. Mechanism three seems to be excluded since the proportion of tri-, di-, and monodeuterioacetate was the same whether or not only a trideuterio precursor or a 1:1 mixture of deuterio and hydrogen precursors was used. Mechanism two, the intramolecular hydrogen transfer, would account for this unaltered proportion of the types of deuterioacetates but an isotope effect would be expected because of the cleavage and reformation of the hydrogen bond. The isotope effect would only be observed, however, if the step was or became a rate-limiting step. Thus the results favor the carbanion mechanism but are not completely conclusive. The intramolecular mechanism or the carbanion mechanism does not account for the formation of di- and monodeuterioacetate from the trideuteriomethyl precursors: these forms may result from secondary exchange reactions.

The bacterium *Clostridium thermoaceticum* performs a total synthesis of acetate from CO_2. The methyl group of the acetate is formed by reduction of CO_2 to formate which is converted to 5,10-formyl-THF[1] and by further reduction to H_3C-THF. The methyl group is then transferred to a cobalt of a corrinoid enzyme yielding a cobalt–methylcorrinoid enzyme complex which is carboxylated to yield acetate (Ljungdahl and Wood, 1969).

We have earlier proposed two mechanisms for the carboxylation step (Ljungdahl *et al.*, 1966) and these are outlined in Figure 1 using deuterated precursors. The first mechanism involving a carboxymethylcorrinoid as an intermediate was proposed by Ljungdahl *et al.* (1968). They isolated a highly radioactive compound from *C. thermoaceticum* after exposure to $^{14}CO_2$ for 15 sec which on photolysis under aerobic conditions yielded radioactive products similar to those obtained by photolysis of synthetically prepared *Co*-carboxymethylcobalamin (Ljungdahl and Irion, 1966). In addition the cleavage of *Co*-carboxymethylcobalamin to acetate was catalyzed in the presence of TPNH by a protein isolated from *C. thermoaceticum* (Ljungdahl *et al.*, 1966, 1967). The second mechanism is based on a suggestion by Ingraham (1964) that H_3C-B_{12} may act as a biological Grignard reagent. A third mechanism has recently been proposed by Schrauzer and Sibert (1970). They suggest that a diol facilitates the reductive cleavage of H_3C-B_{12} with formation of a methyl carbanion, which reacts with CO_2 to form acetate. Evidence for the participation of a thiol in the formation of acetate in extracts of *C. thermoaceticum* has been obtained by Poston *et al.* (1966) who found that the formation of acetate is inhibited by sulfhydryl reagents.

Ghambeer *et al.* (1971) have prepared extracts of *C. thermoaceticum* which effectively convert the methyl group of $H_3{}^{14}C$-B_{12} or $H_3{}^{14}C$-THF to the methyl group of acetate during fermentations of pyruvate or α-ketobutyrate. To further elucidate the mechanism of carboxylation such extracts have been used to convert D_3C-B_{12} and D_3C-THF to acetate. The resulting deuterioacetate has been analyzed in the mass spectrometer. The rate of formation of acetate from D_3C-B_{12} compared to H_3C-B_{12} has been determined as a measure of the deuterium isotope effect.

Methods and Materials

Compounds. D_3C-B_{12} was prepared from cyanocobalamin using trideuteriomethyl iodide (Hogenkamp and Rush, 1965; Mervyn and Smith, 1966). D_3C-THF was synthesized

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[1] THF, tetrahydrofolate; H_3C-THF, 5-methyltetrahydrofolate; D_3C-THF, 5-trideuteriomethyltetrahydrofolate; H_3C-B_{12}, methylcobalamin; D_3C-B_{12}, trideuteriomethylcobalamin; Co(I), cobalt(I); Co(II), cobalt(II); H_3C-Co(I), corrinoid enzyme; H_3C-Co(II), corrinoid enzyme–cobalt(II) enzyme complex.

TABLE II: Types of Deuterioacetate Formed from D_3C-B_{12} or D_3C-THF, during Fermentation of α-Ketobutyrate by an Enzyme Preparation from *C. thermoaceticum*.

Expt No.	Methyl Compound	Non-deuterated Acetate (%)	Deuterated Acetate[a] (%)	Composition of Deuterated Acetate[a] $H_2DCCOOH$ (%)	HD_2CCOOH (%)	D_3CCOOH (%)
I	D_3C-B_{12}	79.2	20.8	11.1	20.7	68.2
II	D_3C-B_{12} + H_3C-B_{12}	80.6	19.4	17.4	23.1	59.5
III	D_3C-THF	82.1	17.9	25.4	25.4	49.2
IV	D_3C-THF + H_3C-THF	88.7	11.3	26.5	23.0	50.5
V	D_3C-B_{12}	77.3	22.7	1.3	4.4	94.3
VI	D_3C-THF	65.9	34.1	16.7	30.8	52.5

[a] These values were determined from the peak heights of *m/e* 60, 61, 62, and 63 and represent the average of seven measurements. There was no measurable ion beam at *m/e* 59, 58, 57, or 56. A sample calculation from the results of the CD_3-B_{12} (expt V) is given. The voltage measurements from the respective *m/e* values were: 60, 0.5444; 61, 0.1498; 62, 0.01002; 63, 0.1507. The contribution of CH_3COOH to *m/e* 61 and 62 is 2.4 and 0.34% of *m/e* 60 due to the normal abundance of ^{13}C and ^{18}O, ^{17}O. The correction is $0.5444 \times 0.024 = 0.01307$ and $0.5444 \times 0.0034 = 0.00239$. These values were subtracted from *m/e* 61 and 62 to give *m/e* 61 = 0.01498 − 0.01307 = 0.00191 and *m/e* 62 = 0.01002 − 0.00239 = 0.00763. The total corrected voltage from *m/e* 60, 61, 62, and 63 thus was: 0.5444 + 0.00191 + 0.00763 + 0.1507 = 0.7046. The per cent composition of the isolated acetate was calculated as follows: H_3CCOOH = (0.5444/0.7046)100 = 77.3, $H_2DCCOOH$ = (0.00191/0.7046)100 = 0.3, HD_2CCOOH = (0.00763/0.7046)100 = 1.0, D_3CCOOH = (0.1507/0.7046)100 = 21.4. The per cent composition of the deuterated acids was calculated for expt V as follows: 0.3 + 1.0 + 21.4 = 22.7 and $H_2DCCOOH$ = (0.3/22.7)100 = 1.3, HD_2CCOOH = (1/22.7)100 = 4.4, D_3CCOOH = (21.4/22.7)100 = 94.3.

reaction as determined by spectrophotometric assay could be accounted for from this source. However, tests with $^{14}CO_2$ have shown that enzyme preparations like those used in these experiments (Gnanamoorthy *et al.*, 1971) do not catalyze a significant total synthesis of acetate from CO_2; CO_2 is fixed in acetate only when the methyl precursor is added and almost exclusively in the carboxyl group.

The source of the acetate appears to have been from endogenous compound(s) present in the crude extract. In the absence of added substrates (α-ketobutyrate and the methyl precursor) about 0.12 μmole of acetate was formed per mg of protein in 90 min at 57°. Thus about 220 μmoles might be formed from this source. Attempts to reduce the endogenous formation of acetate by extensive dialysis of the enzyme preparation were not successful and led to inactivation of the enzyme.

It is noted that the propionate formed from the α-ketobutyrate was much less than a mole for mole yield. The additional products which are formed were not identified but under these conditions, in addition to acetate, acetoin has been observed as a product from pyruvate.

Mass Analysis of the Acetate. The data from the mass analysis of the acetate from experiments with D_3C-B_{12} and D_3C-THF are shown in Table II. The data of columns five to seven show the composition of the acetate based on only those molecules which contained deuterium. These data are presumed to reflect the results which would have been obtained if endogenous acetate had not been produced and diluted the acetate produced from the methyl precursors.

The results from only those molecules which contained deuterium show that from 49 to 94% of the dideuterioacetates contained three deuterium with a much higher value for D_3CCOOH in expt V than in any of the other experiments. The latter conversion is almost quantitative since the methyl moiety was 95.3% trideuterio. There were substantial amounts of both mono- and dideuterioacetates formed in expt I-IV and VI.

Test for Exchange of the Methyl Hydrogen of Acetate during Incubation with Enzyme. An enzyme preparation, similar to those used in the deuterium experiment, was tested for capacity of catalyzing an exchange of methyl protons of acetate. Such an exchange would give rise to mono- or dideuterioacetate. For this purpose an enzyme preparation containing 128 mg of protein was incubated for 2 hr at 55° under N_2 with 0.5 μmole of tritiated acetate containing 416,500 cpm in a total volume of 6 ml. The incubation contained 600 μmoles of potassium phosphate (pH 7.0), 24 μmoles of cysteine, 150 μmoles of sodium α-ketobutyrate, and 15 μmoles of ferrous ammonium sulfate and similar incubations were done in absence of enzyme or without the α-ketobutyrate or ferrous ammonium sulfate. After the incubation an aliquot part of the mixture was passed through a small Dowex 2 (50-100 mesh) column which retained the tritiated acetate, while tritium in the water passed through the column. The total amount of tritium found in the water from the incubations in the presence of enzyme varied from 3000 to 6000 cpm, while that from the control without enzyme was 4200 cpm. The acetate was recovered from the incubations by steam distillation and chromatography on Celite and contained from the nonenzyme incubation 416,900 cpm, while from the enzyme incubations 406,000-421,000 cpm. It is concluded that acetate once formed was stable and that the enzyme preparation did not catalyze an exchange of the methyl hydrogens of acetate and water, nor some loss of hydrogen by side reaction involving the methyl hydrogens of acetate.

Tests for Isotope Effect on the Conversion of Methyl-B_{12} to Acetate. If the conversion of methyl-B_{12} to acetate involved the cleavage of a carbon-carbon or a hydrogen bond from the methyl group as indicated in Figures 2 or 3, there might be an isotope effect and the acetate would be formed at a lower rate

TABLE III: Test for an Isotope Effect on the Conversion of Methyl-B_{12} to Acetate.[a]

Time (min)	I Endogenous (μmoles/ml)	II α-Keto-butyrate (μmoles/ml)	III α-Keto-butyrate + CH_3-B_{12} (μmoles/ml)	IV α-Keto-butyrate + CD_3-B_{12} (μmoles/ml)
30	0.69	0.23	0.67	0.62
60	1.29	0.76	1.52	1.65
90	1.62	1.12	2.37	2.25

[a] The acetate at zero time in micromoles per milliliter was in I, 0.052; II, 0.056; III, 0.074; IV, 0.106. These values have been subtracted from the values shown in the table. The reaction mixture contained in 3 ml 60 mg of protein, 0.03 M $Fe(NH_4)_2SO_4$, 0.001 M thiamine pyrophosphate, 0.03 M $KHCO_3$, and where indicated 0.1 M α-ketobutyrate, 1.1 mM CH_3-B_{12}, and 1.5 mM CD_3-B_{12}.

from D_3C-B_{12} than from H_3C-B_{12}. A comparison of the rates of acetate formation from these substrates is shown in Table III. In addition the rates of formation of acetate are given when α-ketobutyrate but no methyl-B_{12} was added to the enzyme preparation and also without substrates (endogenous formation of acetate). There was a substantial formation of endogenous acetate (expt I) and when α-ketobutyrate was added (expt II) the production of acetate was somewhat lower than the endogenous rate. This inhibition by α-ketobutyrate has been observed repeatedly. Addition of methyl-B_{12} increased the rate of formation of acetate and the increase was the same with H_3C-B_{12} (expt III) as with D_3C-B_{12} (expt IV). Thus there was no observed isotope effect in the formation of acetate from D_3C-B_{12}.

Discussion

The use of D_3C-B_{12} and D_3C-THF appeared to be an attractive tool to distinguish between the carboxymethyl and the Grignard type of mechanism (or any other mechanisms involving a methyl carbanion) for the formation of acetate (Figure 1). If the reaction occurred according to the carboxymethyl mechanism one would expect the formation of dideuterated acetate, while according to the Grignard mechanism trideuterated acetate would be the product. However, the results of these experiments could only have been conclusive

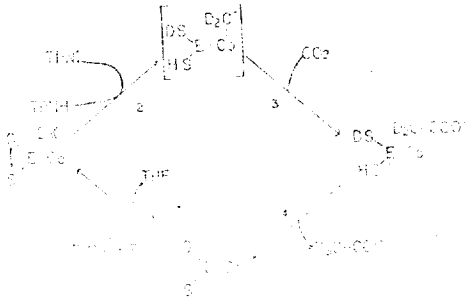

FIGURE 2: Formation of trideuterioacetate via a carboxymethyl-corrinoid involving intramolecular hydrogen transfer through a disulfide of the enzyme.

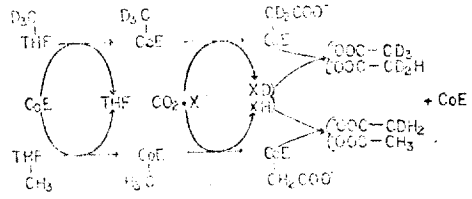

FIGURE 3: Formation of tri-, di-, and monodeuterioacetate via a carboxymethylcorrinoid involving intermolecular hydrogen transfer mediated by a hydrogen carrier X.

if dideuterioacetate was the product, since it is possible to form trideuterioacetate by either mechanism. For example, the hydrogen atom (or deuterium) in reaction 1 (Figure 1) may not leave as a proton freely exchangeable with the solvent; instead it may combine with a carrier, *e.g.*, a disulfide as shown in Figure 2. The hydrogen atom may then be added back to the same carbon from which it was abstracted by an intramolecular reaction. The disulfide mechanism in Figure 2 was chosen because Poston *et al.* (1966) found that the formation of acetate from CH_3-B_{12} is inhibited by iodoacetate and cadmium which react with vicinal sulfhydryl groups. Normally hydrogens on sulfhydryl groups exchange with water rapidly but if the reaction occurred in a hydrophobic region of the enzyme the exchange might be prevented or be slow and there would be retention of deuterium during the reaction.

A second hypothesis, involving a carboxymethyl intermediate, is outlined in Figure 3 which involves an intermolecular reaction. The hydrogen is abstracted from the methyl group by a carrier X, which is not tightly bound to the enzyme. Thus when the reaction is with a mixture of D_3C-THF and H_3C-THF a pool would be formed of XD and XH. The "hydrogen" added back in the formation of acetate would be either a D or an H. Consequently a mixture of tri- and dideuterioacetate would be formed from D_3C precursors, while H_3C precursors would yield mono- and nondeuterioacetate via the intermolecular reaction. The purpose of presenting these schemes is only to illustrate the effect of mechanisms involving (Figure 1) loss of D as a deuteron and (Figures 2 and 3) retention of D by an intramolecular and intermolecular transfer. Other mechanisms illustrating these points may be just as plausible as those chosen.

The result of our experiments was that the predominant acetate formed from the trideuterated methyl donors was trideuterioacetate. Thus there was retention of all three hydrogens of the methyl group during the carboxylation reaction. From Table II it can be seen that more trideuterioacetate was formed from the D_3C-B_{12} than from D_3C-THF and this was especially evident in expt V. These results seem to favor the Grignard-like mechanism involving a methyl carbanion intermediate, or the intramolecular carboxymethyl mechanism (Figure 2). However, the relatively high amounts of monodeuterioacetate and dideuterioacetate could indicate a mechanism involving an intermolecular transfer of hydrogen as illustrated in Figure 3. The experiments with 1:1 mixtures of trideuterated and nondeuterated methyl precursors were designed to distinguish between mechanisms. It would be expected that the ratios of the deuterioacetate species would differ with a 1:1 mixture of precursors formed from [illegible] D_3C-THF or D_3C-THF [illegible] would be the intermolecular mechanism. The data in Table II show that the addition of H_3C-B_{12} and H_3C-THF to the D_3C-B_{12} and D_3C-THF did not result in a change in the ratio of the mono-, di-, and trideuterioacetate. The high rate of endogenous acetate synthesis may indicate a large pool

catalytic hydrogenation of folic acid with PtO_2 followed by condensation of the resulting tetrahydrofolate with D_2-formaldehyde and reduction to D_2C-THF by $NaBD_4$ as described by Sakami (1963). The D_2C-THF was purified by column chromatography on QAE-Sephadex (Parker *et al.*, 1971) and stored in sealed evacuated vials. All other chemicals were commercial products.

The isotopic purity of the D_3C-B_{12} was not determined directly with the mass spectrometer; instead the D_3CI from which it was synthesized was examined. The results were D_3CI, 95.3%; HD_2CI, 3%; H_2DCI, 1%; and H_3CI, 0.7%. The deuterium content of D_3C-THF was estimated by nuclear magnetic resonance (nmr) spectrometry in D_2O. Methyltetrahydrofolate has a peak at τ 7.5, that is characteristic of the hydrogen of the 5-*N*-methyl group. When the D_3C-THF was assayed the peak at τ 7.5 was absent, indicating that the 5-methyl group was composed of greater than 90% of D_3C groups.

Procedure. Enzyme preparations from *C. thermoaceticum* were precipitated with ammonium sulfate and dialyzed as described by Ghambeer *et al.* (1971). α-ketobutyrate was chosen as the substrate since it is converted to propionate and thus does not dilute the deuterium atom bound from D_3C-B_{12} or H-C-T-37. The α-ketobutyrate was determined before and after fermentation in deproteinized portions by use of lactate dehydrogenase and the acetate by the spectrophotometric method described by Schulman and Wood (1971). Deproteinization was done by addition of H_2SO_4 to give pH 1. The amount of methyltetrahydrofolate utilized during the fermentation was estimated by determination of the THF liberated in the process of methyl transfer. The assay involved the oxidation of the THF to the methenyl derivative by addition of acid and heating for 5 min at 100° (Rosenthal *et al.*, 1955). The resulting methenyltetrahydrofolate was measured spectrophotometrically at 350 nm. Protein was assayed according to Gornall *et al.* (1949).

It was found that the amount of methyl-B_{12} converted to B_{12} could not be determined by spectral change since the B_{12} was absorbed on the protein during the deproteinization and could not be recovered quantitatively. By use of a tracer amount of $H_3{}^{14}C$-B_{12} the conversion of the methyl of H_3C-B_{12} to the methyl of acetate was determined by measuring the radioactivity in the acetate as described by Ghambeer *et al.* (1971). The ^{14}C was determined by the liquid scintillation technique.

The acetate was separated from the deproteinized solution by continuous extraction with ether for 48 hr. The acid in the ether extract was titrated with 0.2 N sodium hydroxide every 8 hr to determine that the extraction was complete. The resulting extract was evaporated to dryness, dissolved in 2 N H_2SO_4, and applied to a column of 10 g of Celite 545 (Swim and Krampitz, 1954). The propionic acid which emerged first from the Celite column was determined by titration. Those fractions which contained acetic acid also contained α-ketobutyric acid. These fractions from expt I–IV were titrated with 0.2 N NaOH and were pooled, taken to dryness, redissolved in 1.0 ml of water, and treated with 10 ml of saturated ceric sulfate (in 10 N H_2SO_4) followed by 5 ml of 5 N dichromate. The α-ketobutyric acid is oxidized to propionic acid by ceric sulfate (Sachs, 1957) and the dichromate oxidizes some other organics which might be present. The oxidized solution consisted of formic acid, acetic acid, and was separated from the propionic acid resulting from the oxidation of α-ketobutyric acid by chromatography on a second Celite column. The neutralized acetic acid fractions were combined and

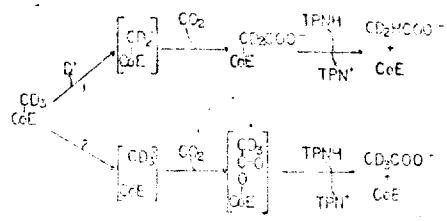

FIGURE 1: The carboxymethyl mechanism (1) and the Grignard mechanism (2) for formation of acetate from methyl-B_{12} and CO_2 and proposed use of trideuteriomethyl precursors to differentiate between the mechanisms.

dried, and the amount of deuterioacetate was determined by mass analysis. In expt V and VI the acetic, α-ketobutyric acid fraction of the first Celite column was oxidized with 25 ml of the ceric sulfate solution exclusively instead of the combination of ceric sulfate and dichromate.

Mass Analysis of Deuterioacetate. The acetate was dissolved in H_2O and approximately 100 μmoles in no more than 0.2 ml of solution was placed in a clean dry 10-ml test tube with a ground-glass stopper. Concentrated HCl^* (10–15 μl) was added so that the resulting pH was approximately 1. Anhydrous diethyl ether (1 ml, distilled and stored over sodium wire in a dark bottle) was added; the tube was stoppered and shaken for about 2 min to equilibrate the phases. The aqueous phase was removed with a Pasteur pipet and the ether phase, which contained about 60% of the original acetic acid was shaken with two portions of P_2O_5 (about 10 mg). The ether solution was placed in a 0.5 × 9 cm test tube fitted with a high vacuum stopcock and a 10/30 ground glass joint for attachment to the mass spectrometer. The main portion of the ether was removed at −78° (Dry Ice–propanol) on a vacuum system and the rest at −78° by the pumping system of the mass spectrometer which was a cycloid type (Model 21-130). After being evacuated to a pressure of 1×10^{-7} mm, the frozen acetic acid mixture was allowed to warm. Under these conditions the entire sample was vaporized and introduced into the sample inlet system to give pressures of 30–80 μ measured by a flexible membrane micromanometer. The sample was introduced into the mass spectrometer through a gold foil leak and ionized at 70 eV and 20 μA of filament current. Voltage measurements to five decimals of *m/e* 60, 61, 62, and 63 corresponding to H_3CCOOH, $H_2DCCOOH$, HD_2CCOOH, and D_3CCOOH were determined using a John Fluke differential voltmeter connected across the output of the amplifier. The data are presented as the per cent composition of the types of deuterated acetate.

Conditions for the Enzyme Reaction. The experiments of Table I were carried out in 125-ml conical flasks. The enzyme preparation, α-ketobutyrate, ferrous ammonium sulfate, thiamine pyrophosphate, and sufficient water to give the desired volume were added at 4°. The flask was stoppered with a serum cap, evacuated through a syringe needle, and then flushed with argon. The process was repeated five times at 4°. The $KHCO_3$ and the methyl substrates were then injected with a syringe, the contents were mixed, and incubation was at 57°. Small aliquot samples were withdrawn with a syringe and were analyzed for utilization of $H_3{}^{14}CI$ and $H_3{}^{14}CTHF$ by methods described above. At the end of the incubation the solution was acidified with sulfuric acid to pH 1. The gas phase was sampled at 70°C for 30 min, and the supernatant solution was used for isolation of the acetate.

TABLE I: Formation of Acetate from Deuteriomethyl-B_{12} and from Deuteriomethyl-THF by an Enzyme Preparation from *C. thermoaceticum* Fermenting α-Ketobutyrate.[a]

Experiment		I D_3C-B_{12} (μmoles)	II D_3C-B_{12} H_3C-B_{12} (μmoles)	III D_3C-THF (μmoles)	IV D_3C-THF H_3C-THF (μmoles)	V D_3C-B_{12} (μmoles)	VI D_3C-THF (μmoles)
Methyl compound utilized			85	128	134		
Acetate by spectroassay	Final	365	371	368	336		
	Initial	16	16	34	35		
	Net	349	355	334	301		
Acetate isolated		575	566	880	824	290	264
α-Ketobutyrate utilized		2410	2370	2310	2180	2900	2195
Propionate formed		787	845	670	776	1100	710

[a] The results are given in μmoles/100 ml of fermentation mixture. The volume in expt I, II, and V was 120 ml with 1950 mg of protein in expt I and II and 1620 mg in expt V and contained 0.1 M potassium phosphate (pH 7.0), 4×10^{-5} M cysteine, 0.05 M α-ketobutyrate, 0.003 M ferrous ammonium sulfate, 0.001 M thiamine pyrophosphate, and 0.03 M $KHCO_3$, and in expt I 200 μmoles of D_3C-B_{12}, expt II 200 μmoles of methyl-B_{12} containing 50% D_3C-B_{12} and 50% $H_3^{14}C$-B_{12}, and in expt V 220 μmoles of D_3C-B_{12}. Incubations were at 37° and for 80 min (expt I and II) and 135 min (expt V). The volume in expt III, IV, and VI was 100 ml with 1840 mg of protein in expt III and IV and 1500 mg in expt VI. The concentrations of other components were the same as in experiments I, II, and V except the methyl-B_{12} was replaced by 400 μmoles of DL-D_3C-THF in expt III and VI and by 400 μmoles of DL-methyl-THF containing 50% D_3C-THF and 50% H_3C-THF in expt IV. Incubations were at 37° and for 110 min (expt III and IV) and for 90 min (expt VI).

The experiments of Table III were done similarly but in a volume of 3 ml in test tubes. In these experiments the rates of the formation of acetate from D_3C-B_{12} or H_3C-B_{12} were measured to determine if there was an isotope effect.

Results

Six experiments for mass analysis of the acetate were done with the deuteriomethyl compounds, two with D_3C-B_{12}, one with an equal mixture of D_3C-B_{12} and $H_3^{14}C$-B_{12}, two with D_3C-THF, and one with an equal mixture of D_3C-THF and H_3C-THF. The purpose of using mixtures of deuterated and nondeuterated methyl compounds was for the detection of intermolecular hydrogen transfer as will be considered in the Discussion.

Products of Fermentation. The progress of the conversion of the $H_3^{14}C$-B_{12} to acetate in expt II was determined by removal of samples at 30, 50, and 80 min. The radioactivity in the isolated acetate was 2800, 7740, and 7640 cpm, respectively, from 1 ml of the fermentation mixture. Apparently the utilization of $H_3^{14}C$-B_{12} stopped after 50 min. About 50% of the methyl-B_{12} had been utilized at this time. The yield of THF from the methyl-THF was 0.4, 0.77, and 1.28 μmoles per ml in expt III at 30, 50, and 110 min. It was 0.66, 0.96, and 1.34 in expt IV at the same times. These amounts are equivalent to 64 and 67%, respectively, of the available methyl-THF if it is assumed that only one isomer is utilized.

The yields of acetate, amounts of methyl compounds and [illegible] shown in Table I, indicate that α-ketobutyrate [illegible] to acetate formation [illegible] from the results of Table I that there was a very substantial yield of acetate which did not originate from the methyl precursors. The amounts of methyl precursors utilized were 85, 128, and 134 μmoles, respectively, in expt II, III, and IV but the net acetate formed as determined by spectrophotometric assay varied from 301 to 355 μmoles for expt I–IV. Furthermore, during the isolation of the acetate from these experiments there was additional formation of acetate since the isolated acetate varied from 566 to 880 μmoles. Thus in expt II 85% of the acetate [566/(85/55%)] is estimated to have been formed from non-methyl precursors, and in expt III and IV 86 and 84%, respectively. The source of the acetate which was formed during the isolation *per se* is unknown. The fractions from the first Celite column which contained the acetate and α-ketobutyrate were oxidized with ceric sulfate and dichromate to remove the α-ketobutyrate. There may have been unknown compounds present in these fractions which were oxidized to acetate. Possibly the ether used in the extractions contained contaminants. During the isolation of acetate from expt V and VI, the oxidation was performed with ceric sulfate only and this was done carefully to avoid excess of oxidant. In these experiments much less acetate was isolated, which seems to indicate that at least some of the acetate in expt I–IV was formed during the oxidation. Unfortunately the acetate formed during the incubations in expt V and VI was not determined.

The amount of α-ketobutyrate utilized during the fermentation was substantial, 2180 to 2900 μmoles. The fermentation is considered to occur in whole cells as follows

$$4CH_3CH_2COCOOH + 4H_2O \longrightarrow 4CH_3CH_2COOH + 4CO_2 + 8H$$

$$8H + 2CO_2 \longrightarrow CH_3COOH + 2H_2O$$

sum:

$$3CH_3CH_2COCOOH + 2H_2O \longrightarrow 4CH_3CH_2COOH + 2CO_2 + CH_3COOH$$

Accordingly, unlabeled acetate would be formed during the fermentation by biosynthesis of acetate from CO_2 and a substantial amount of acetate which was formed during the fer-

nondeuteriomethyl precursors and therefore the effect of added H_3C-B_{12} or H_3C-THF would be masked. However, this is not likely since extracts used in these experiments do not catalyze fixation of a substantial amount of $^{14}CO_2$ when incubated alone or in the presence of α-ketobutyrate or pyruvate (Ghambeer *et al.*, 1971). Hence the endogenous acetate appears to be formed by a pathway which does not involve CO_2 fixation and H_3C-THF or H_3C-B_{12}. Apparently the endogenous acetate is formed *via* pyruvate formed from substrates present in the enzyme preparation. The fact that α-ketobutyrate inhibits the endogenous acetate formation (Table III) is in agreement with this assumption. It is concluded from the results of the experiments with raised deuterio and hydrogen precursors that a carboxymethyl mechanism is excluded which involves an intermolecular hydrogen transfer. The fact that D_3CCOO^- was the predominant product in all experiments also makes the occurrence of an intermolecular hydrogen transfer unlikely.

Retention of all three hydrogens of methyl-B_{12} and methyl-THF during the carboxylation step suggests the mechanism is *via* the carbanion or a reaction involving an intramolecular hydrogen transfer. In the latter mechanism a hydrogen carbon bond is broken twice, first in the transfer from the methyl group to the enzyme-bound carrier and second when the hydrogen atom is transferred back to the methyl group of the acetate (Figure 2). It is well known that mechanisms involving hydrogen carbon bond cleavage generally exhibit an isotope effect. For example, in the reaction catalyzed by propanediol dehydrase (conversion of propanediol is converted to deuteriated propionaldehyde at rates [illegible] the rate of the nondeuterated substrate (Frey *et al.*, 1968). Similarly the deuterium isotope effect in the cobalamin-ammonia-lyase reaction is 7.4 (Weisblat and Abeles, 1971). Both these reactions involve two hydrogen carbon bond cleavages. We did not observe an effect on the rate of acetate formation from D_3C-B_{12} compared to the nondeuterated substrate. However, the isotope effect may not be evident because the cleavage of the hydrogen carbon bond may not be the rate-limiting step; such a step might well be the carboxylation reaction.

If the acetate is formed by a carbanion mechanism, the formation of di- and monodeuterioacetate must occur by secondary reactions, since only trideuterioacetate is formed by this mechanism. A possible explanation of the formation of mono- and dideuterioacetate is that the D is lost by exchange reactions. Such exchange might occur by reversible oxidation of 5-methyltetrahydrofolate to 5,10-methylenetetrahydrofolate and also to 5,10-methenyltetrahydrofolate. The result would be formation of mono- and dideuterio-5-methyltetrahydrofolate, which would be converted to mono- and dideuterioacetate. The enzymes 5,10-methylenetetrahydrofolate reductase and dehydrogenase, which catalyze these reactions, are both present in *C. thermoaceticum* as well as *Clostridium formicoaceticum* (O'Brien *et al.*, 1971, and unpublished results). The latter organisms also perform the synthesis of acetate from CO_2 by a mechanism similar to that of *C. thermoaceticum* (O'Brien and Ljungdahl, 1972; Schulman *et al.*, 1972). Since there was formation of mono- and dideuterioacetate with trideuteriated methyl-B_{12}, it would be necessary in this case to assume that the methyl group of D_3C-B_{12} is converted to D_3C-THF which then is subject to the above reversible reactions. The fact that more di- and monodeuterioacetates are formed from D_3C-THF than from D_3C-B_{12} may support the above assumption. The formation of 94% D_3C-CO_2H in exp. V may have resulted because for unknown reasons the methyl group of D_3C-B_{12} was not transferred to THF to form D_3C-THF in this experiment. Another possibility is that there is an exchange reaction which occurs at the site of the acetate formation. An exchange at the corrinoid site is proposed by Penley *et al.* (1970) to account for the formation of methane from difluoromethylcobalamin by methane bacteria.

It is of interest that formation of methane from acetate by *Methanosarcina barkeri* occurs exclusively from the methyl carbon and that all hydrogens are retained (Pine and Barker, 1956). It has been suggested that the methane formation is *via* a methylcorrinoid and that it occurs in a manner which is the reverse of acetate formation (Stadtman, 1967). Our results showing retention of the hydrogens during formation of acetate seem to support this idea. Furthermore, Kislid. (1963) has studied the formation of methionine in *Escherichia coli* and demonstrated with tritium labeling that the methyl group of H_3C-THF is transferred intact to homocysteine. This reaction also involves a methyl corrinoid as intermediate.

In conclusion it might appear, since D_3CCOO^- is the predominant product, that the Grignard or a similar reaction is the most probable mechanism. However, it is well known from studies of other reactions catalyzed by corrinoid enzymes that it is characteristic of these enzymes that there is hydrogen transfer without exchange with the protons of the water. One exception is the ribonucleotide triphosphate reductase reaction. These studies have been reviewed extensively (Barker, 1967; Hogenkamp, 1968; Stadtman, 1971). Our results do not exclude a carboxymethylcorrinoid as intermediate, but any mechanism proposed must account for the formation of trideuterioacetate.

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ITALIAN TRANSLATION

HEALTH INJURIES CAUSED BY ACETIC ACID IN THE PRODUCTION OF CELLULOSE ACETATES

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Acetic acid (CH_3COOH) is a colorless liquid with a pungent odor, which solidifies at 16.7°C in the form of crystals similar to ice and boils at 118°C; its density is rather close to that of water, it burns with a bluish flame, and it can be mixed in all ratios with water and alcohol with liberation of heat and contraction in volume. Acetic acid was prepared at one time by oxidation of slightly alcoholic liquids by means of aerobic microorganisms (Acetobacter) and is obtained at present in this manner in the production of vinegar. On an industrial scale, it is prepared by hydration of acetylene followed by oxidation of acetaldehyde, or as a by-product of wood distillation by treatment of sodium pyrolignite with sulfuric acid. It is widely used in the chemical industry in the production of its salts and as a solvent for some synthetic resins, nitrocellulose and celluloid, in the textile industry for the production of cellulose acetates, for dyeing wool and silk with alizarine dyes, in the pharmaceutical industry, in the production of vinegar, etc.

In spite of the fact that workers are exposed to acetic acid every day, as a result of all these operations in modern industry, no publications on the hazards and occupational pathology of this product can be found in the field of industrial medicine of the past 30 years; published reports contain data obtained during the first decades of this century, which

attribute to acetic acid, during occupational exposure, an irritating action on conjunctival mucosas and those of the upper airways, and also on the skin. Intensive and prolonged exposure to acetic acid can cause conjunctivitis, palpebral edema, and in rare cases keratitis, bronchitis, dental erosions, slight intestinal disorders, weight loss and pallor. More extensive are the reports concerning lesions caused by ingestion of the product (either accidental or for suicide purposes); these include severe, ulcerative-necrotic lesions of the oropharynx and of the upper digestive tract, accompanied by intense retrosternal pain, vomiting of blood, diarrhea, dyspnea, collapse, and in some cases anuria followed by uremia. A few years ago, Ravera has described a case of chemical pneumonia with scattered foci, cured after about 10 days, in a woman who was forced to inhale vapors of concentrated acetic acid for a few minutes in order to reanimate her during the course of syncope.

Toxicological data are also quite scarce; according to Flury and Zernik, 2-3 mg/l of acetic acid exert an immediate disturbing effect on man and cannot be tolerated for more than 3 minutes, while 0.03 mg/l cause an irritation of the nasal mucosa. In animals, 30-36 mg/l have the same effect, while a concentration of 47-86 mg/l causes a tolerable hyperemia of the tracheal mucosa without any serious disorders. For years, the maximum permissible concentration for a daily 8 hour exposure was considered to be 0.025 mg/l, equal to 10 ppm.

In view of the small number of reports published so far and the new interest which this compound has aroused in our country as a result of the legislative table published on 15 November 1952, which insures diseases caused by acetic acid, we are prompted to publish some of our observations made on a group of workers in the acetylation section of a plant for the

production of cellulose acetates, who are daily exposed to significant concentrations of acetic acid.

At the plant visited by us, acetylation of cellulose is carried out in a large 2-story high building, with communications between the 2 floors, by the following process: 4 rotating copper reactors are loaded manually with cellulose, and concentrated acetic acid is then fed through pipes into the reactors at 60°C, to give cellulose acetate. The rotary reactors are under suction pressure, and the suction valves are mounted on top of the charge and discharge openings (manholes); three charge operations are performed in each working cycle. In spite of the fact that suction devices are available and are regularly used during charging and sampling operations, a frequent and intensive escape of acetic acid vapors takes place, either as a result of the malfunctioning of suction valves or the imperfect sealing of many equipment joints, due to the corrosive action of acetic acid. The reactors are discharged at the bottom through manholes, and the cellulose acetate solution is fed into a saponification tank, where 60% acetic acid is added to obtain cellulose diacetate; here, the solution is precipitated and transferred to the ripening tank, where it is allowed to stand for 2-3 hours. After ripening, acetic acid is removed by means of vacuum filters. The cellulose diacetate is automatically discharged onto other vacuum filters, washed with water, filtered again, and manually discharged into a press where it is dried; prior to milling it is transferred to drying ovens and from there packed into jute bags. This second phase of the process does not expose workers to the inhalation of acetic acid vapors.

In the same building, an operation is performed, which although it is not continuous, constitutes a source of dispersal of large amounts of acetic acid; this operation involves the filtration of cellulose triacetate, used

in the manufacture of special photographic film, and is carried out in 2 filter presses located on the ground floor of the plant. During replacement of the filter cloth, lasting about 30 minutes (10 minutes for removing the cloth from the filter, and 20 minutes for mounting the cloth on the filter), considerable amounts of acetic acid vapors escape from the cloth supplied at a rate of 75 m/min by a belt located under the filter press, and only a minimal portion of these vapors is caught by the suction fan. Moreover, the workers replacing the cloth handle the replacement cloth with bare hands.

Workers are equipped with goggles and a face mask with a cotton filter; during work, a small bassin filled with a bicarbonate solution is located nearby, in which the workers often wash their hands.

To determine the concentration of acetic acid in the atmosphere, we have carried out 6 samplings: the first 3, during normal working conditions, and the other 3 during the operations where acetic acid is dispersed in the ambient air. The results of such samplings are shown in Table 1.

TABLE 1

Concentrations of acetic acid present in the atmosphere of the section where cellulose acetate is produced.

N.	Posizione del prelievo	mg/l	p.p.m.
1	Fra due reattori durante un carico di acido acetico	0,183	75
2	Fra due reattori durante la reazione	0,200	80
3	Fra due reattori al piano di scarico	0,066	26
4	Scarico del acetato di cellulosa	0,348	139
5	Asportazione del filtro del triacetato di cellulosa	0,670	260
6	Carico di acido acetico glaciale e al 60%, durante la determinazione della viscosità.	0,232	92

1. Sampling point

1-Between 2 reactors during an acetic acid charge
2-Between 2 reactors during the reaction
3-Between 2 reactors at the discharge level
4-Cellulose acetate discharge
5-Cloth removal from the cellulose triacetate filter
6-Charging of glacial and 60% acetic acid during viscosity determination

The acetic acid, captured by bubbling air through a sodium hydroxide solution, was determined in all samples by the following 2 methods: 1) by titration of the sodium hydroxide with N/100 sulfuric acid, and 2) by the Vogel lanthanum nitrate colorimetric method.

Under normal working conditions, the acetic acid concentrations are 3-6 times greater than the maximum permissible concentration; during the discharge of acetate from the reactors and the removal of cloth from the cellulose triacetate filter press, the acetic acid concentrations exceed the maximum permissible concentration by 9 to 26 times.

Workers in that section were subjected to a medical examination, and the following results are reported.

1) S.M., 38 years old, worked for 7 years in the acetylation unit, and often also worked on the filter press. Complains of burning of the eyes, throat and hands, especially during work on the filter press, also complains of difficult digestion. Objective symptoms include a thickened, blackened skin on the palm of the hands, furrowed by numerous superficial chaps (crevices) painful to the touch; the conjunctival and pharyngeal mucosas have an intense red coloring, and in the thorax a very harsh breathing with prolonged expiration was noted.

2) R.M., 37 years old, worked for 7 years in the acetylation unit, and also frequently on the filter press. Complains of light burning of the eyes and throat during work. For 4-5 years, has suffered from coughs and catarrh, which are more intense in winter; several months ago, has also noted a pyrosis (heartburn) and some epigastric pain. Objective symptoms include a very dry, creviced and blackish skin on the palm of the hands, a reddish and edematous conjunctival and pharyngeal mucosa, a very harsh

breathing in the thorax with prolonged expiration, some rale (rattling) and hissing especially in the basal areas, and a slight tenderness on epigastric palpation.

3) B.A., 37 years old, worked for 11 years in the acetylation unit and almost every day on the filter press. Compalins of burning of eyes, nose and throat during work, and sometimes of heartburn after meals; for several months, has been suffering from coughs and catarrh, accompanied by a moderate exertional dyspnea. Objective symptoms include dry, blackish, calloused and creviced skin on the palms of hands, intense conjunctival and pharyngeal congestion with hypertrophic and cryptic tonsils, a moderate basal hyperphonesis in the thorax with bases not greatly mobile, harsh breathing with prolonged expiration and numerous rales and hissings during inhalation and exhaling.

4) L.M., 41 years old, worked for 12 years in the acetylation unit and sometimes also on the filter press. Compains of burning of eyes and throat during work, and states that occasional small skin wounds heal very slowly (slow cicatrisation). Objective symptoms include blackish and dry skin on hands, with work calluses (corns) and inital palmar contraction, blackened incisor teeth with some erosion patches, intense reddening and hypertrophy of lymphatic nodules in the conjunctival and pharyngeal mucosas, a diffuse harsh breathing in the thorax, a rhythmic and hard 60 pulse, an arterial pressure of 155-100, and symptoms of a general (widespread) artherosclerosis.

5) P.L., 45 years old, worked for 12 years in the acetylation unit and occasionally on the filter press. Compains of burning of eyes and throat, sometimes of heartburn and constipation, and also of slow cicatrisation (healing) of wounds. Objective symptoms include a very rough and fissured skin of the hands, some erosion patches on incisor and canine teeth, intensely reddened

conjunctival and pharyngeal mucosas, a very diffuse and harsh breathing in the thorax, and a slight tenderness on epigastric palpation.

The 5 workers mentioned above had worked for 7-12 years in an environment where acetic acid is always present in concentrations which sometimes are 8 times greater than the maximum permissible concentration, and occasionally, during work on the filter presses or in case of leaks due to a rupture of values or other parts of the equipment, can even reach values that are 20-30 times higher than the maximum permissible concentration. These workers complain of disorders and exhibit lessions of the skin, of the conjunctival and oral-pharyngeal mucosas, of the respiratory tract, of teeth, and of the digestive apparatus. The most clearly evident alterations of the skin are noted on the palm surface of the hands; the skin here is intensely blackened, dry, furrowed by numerous and deep crevices (chaps), sometimes painful under pressure; wounds exposed to the action of acetic acid are easily transformed into slowly-growing sores with a gray-blackish background. The conjunctivas are hyperemic, there is a copious lacrymal secretion, but in no case were corneal alterations (lesions) observed. The oral pharyngeal cavity is even more hyperemic, and in addition to being intensely red-colored, often exhibits an edematous imbibition of the submucosa, which is more evident on the pillars and the velum pendulum. An hypertrophy of the submucous follicles is not rare, up to the stage of a typical granular pharyngitis; a serous, and in some cases a seromucous, secretion is always observed. Pathological findings were also noted in the thorax, sometimes in the form of a chronic catarrhal bronchitis and sometimes as a chronic asthmatic bronchitis, with an initial pulmonary emphysema in one case. For technical reasons, we were unable to perform a gastric probe or a radiological examination of the digestive tract; 2 out of the 5 workers examined complained of heartburn after meals, occasionally accompanied by epigastric pain and constipation, tenderness

to epigastric palpation. The teeth were sometimes blackened with some small erosion patches on the vestibular surface of the incisors and canines.

As a treatment of conjunctival and pharyngeal lesions, we suggest a frequent ocular washing and inhalations of bicarbonate solutions, for bronchitis the use of alkaline aerosols and bronchodilators, and other symptomatic drugs, and in gastritis the use of antacids and an adequate diet, for the skin the application of eutrophic creams and, in case of hyperkeratosis, the use of keratolytic ointments. For the purposes of prevention, we suggest the adoption of acid-resistant barrier creams for the skin, and we insist on the necessity of using technical measures for protecting the organism against too high ambient concentrations of acetic acid. For this reason, we suggest that maintenance work be performed more accurately and that more extensive repairs be carried out in case of leaks, as well as the strengthening of suction and exhaust equipment and the enclosure of the cellulose triacetate filter in an adjacent building; we also suggest the use of bicarbonate solutions in the form of aerosols during the mounting and removal of cloth on the filter press. The use of face masks equipped with charcoal filters or alkaline absorption agents should be considered only as a temporary measure under exceptional circumstances.

Our findings also allow us to draw some considerations of a more general interest. It is evident that, during continuous exposure, high concentrations of acetic acid are tolerated fairly well from a subjective standpoint; since, in some operations, acetic acid concentrations are present which are 26 times greater than the maximum permissible concentration, the workers will be able to continue performing their work without much discomfort. On the other hand, if we compare the subjective state of the workers with the disorders established by us during our study of working conditions in the acetylation section, we have good reasons to assume that continuous exposure of the workers to acetic acid results in a

certain habituation of the workers towards the effects of this acid. From an objective standpoint, the prolonged action of acetic acid in concentrations considerably higher than the maximum permissible concentration results in a damaging effect and, in the long run, is capable of causing, primarily by means of irreversible lesions of the air tract, a permanent reduction of the working ability. The present value of the maximum permissible concentration appears to have been established with considerable caution and can probably be exceeded to a certain extent without causing any appreciable damage to the health of the workers. There are no specific clinical symptoms or laboratory tests which would permit establishing the occupational nautre of the individual symptoms described here. Therefore, in this connection, the following factors should be used as a basis: 1) The determination of ambient acetic acid concentrations which are continuous and somewhat greater than the maximum permissible concentrations, and 2) the contemporaneous presence of lesions (injuries) in various body systems sensitive to the action of acetic acid. Finally, it is indispensable that workers assigned to work in the presence of acetic acid should undergo a preliminary hiring check-up, in order not to expose to occupational hazards those who are suffering from chronic bronchitis and definite severe injuries of the respiratory apparatus, chronic conjunctivitis and pharyngotonsilitis, gastroduodenitis and active or previous gastroduodenal ulcers, and skin diseases. Periodical medical check-ups, at least once a year, are also advisable.

SUMMARY

Five workers who had been exposed for 7-12 years to atmospheric concentrations of acetic acid up to 0.200 mg/l, i.e. eight times higher than the maximum allowable concentration in normal working conditions, and 0.650 mg/l (26 times greater) during particular working phases, were submitted to medical examination; the findings were: the skin of the hands was considerably blackened hyperkeratosic, furrowed with painful rhagades, the conjunctivas were hyperaemic, no corneal lesions were observed the pharynx was remarkably congested with edema and serous secretion. The examination of the chest revealed: chronic bronchitis, asthmatic-like in 3 cases, initial emphysema in one; the teeth were blackened and showed spots of erosion on the vestibular surface of the incisors and canines; also frequent the disturbances attributable to hyperacid gastritis. It is probably that with continuous exposure the workers can become hardened to the subjective discomfort caused by the acetic acid, whilst the organic lesions may, with the passing of time, be such as to cause a permanent decrease of the working capacity.

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SUI DANNI PER LA SALUTE PROVOCATI DALL'ACIDO ACETICO NELLA PRODUZIONE DEGLI ACETATI DI CELLULOSA

LUIGI PARMEGGIANI e CARLO SASSI

L'acido acetico (CH_3COOH) è un liquido incolore, di odore penetrante, che solidifica a 16°,7 in cristalli che hanno un aspetto simile al ghiaccio, e bolle a 118°; ha una densità assai vicina a quella dell'acqua; brucia con fiamma bluastra; si mescola in tutti i rapporti con acqua ed alcool con sviluppo di calore e contrazione di volume. Si produceva un tempo per ossidazione di liquidi debolmente alcoolici ad opera di microorganismi aerobi (Acetobacter) e così si ottiene tuttora nella produzione dell'aceto; industrialmente si prepara per idratazione dell'acetilene e successiva ossidazione dell'acetaldeide, ovvero come sottoprodotto della distillazione del legno trattando con acido solforico il pirolignito di sodio. È largamente usato nell'industria chimica per la preparazione dei suoi sali, e come solvente di alcune resine sintetiche, della nitrocellulosa, e della celluloide, nell'industria tessile per la produzione degli acetati di cellulosa, per la tintura delle lana e della seta con i colori di alizarina, nella industria farmaceutica, nella produzione dell'aceto, ecc.

Nonostante che tutte queste lavorazioni espongano ogni giorno nella industria moderna molti operai all'acido acetico, non si trovano nella letteratura di medicina industriale degli ultimi trent'anni pubblicazioni riguardanti i rischi e la patologia professionale di questo prodotto; anche i trattati riferiscono dati che risalgono ai primi decenni del secolo, attribuendo all'acido acetico nella esposizione professionale un'azione irritante sulle mucose congiuntivali e delle prime vie aeree, e sulla cute. Per esposizioni intense e protratte si possono avere congiuntiviti, edemi palpebrali, in rari casi cheratiti, bronchiti, erosioni dentarie, lievi disturbi intestinali, dimagramento e pallore. Più ricca è la documentazione riguardante le lesioni provocate dalla ingestione (accidentale o per suicidio) del prodotto: si tratta di gravi lesioni ulcerative-necrotiche dell'orofaringe e del primo tratto del canale digerente, accompagnate da intenso dolore retrosternale, vomito ematico, diarrea, dispnea, collasso, e in qualche caso anuria seguita da uremia. Pochi anni orsono RAVERA ha descritto un caso di polmonite chimica a focolai disseminati, guarita in una decina di giorni, in una donna, alla quale erano stati fatti inalare per pochi minuti vapori di acido acetico concentrato per rianimarla nel corso di uno svenimento.

Anche i dati tossicologici sono molto scarsi: secondo FLURY e ZERNIK 2-3 mg/l provocano nell'uomo disturbi immediati e non possono essere sopportati per più di 3 minuti; 0,93 mg/l determinano irritazione delle mucose nasali; negli animali 30-36 mg/l hanno lo stesso effetto, mentre una concentrazione di 47-86 mg/l è causa di iperemia della mucosa tracheale tollerabile senza gravi disturbi. Da anni si ammette come massima concentrazione tollerabile per una esposizione di otto ore quotidiane quella di 0,025 mg/l, pari a 10 ppm.

La povertà delle notizie sinora pubblicate ed il nuovo interesse attirato su questa sostanza nel nostro Paese dalla tabella legislativa del 15-11-1952, che assicura le malattie causate dall'acido acetico, ci induce a rendere note alcune

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nostre osservazioni su di un gruppo di operai del reparto di acetilazione di una fabbrica per la produzione degli acetati di cellulosa, esposti quotidianamente a concentrazioni rilevanti di acido acetico.

Nello stabilimento da noi visitato l'acetilazione della cellulosa avviene in un ampio fabbricato a due piani, fra loro comunicanti, secondo il seguente schema: quattro reattori rotanti, di rame, sono caricati a mano con cellulosa, poi con acido acetico concentrato, immesso da tubazioni, a 60°; si forma acetato di cellulosa. I reattori rotanti sono sotto aspirazione, e cappe di aspirazione sono impiantate sopra le bocchette di carico e scarico; per turno di lavoro si effettuano di solito tre operazioni di carico. Nonostante la presenza dei mezzi di aspirazione e la regolare messa in funzione di questi durante le operazioni di carico e di campionamento, sia per il cattivo funzionamento delle cappe di aspirazione, sia per la non perfetta tenuta di molti giunti, dovuta all'azione corrosiva dell'acido acetico, si hanno frequenti ed intense fughe di vapore. I reattori sono scaricati dal basso attraverso maniche, e la soluzione di acetato di cellulosa viene inviata nel tino di saponificazione, dove è addizionata di acido acetico al 60% per ottenere il diacetato di cellulosa; quindi è fatta precipitare e messa in fine di maturazione, dove resta per due-tre ore; dopo la maturazione l'acido acetico è eliminato mediante filtri a depressione. Il diacetato di cellulosa è scaricato automaticamente in altri filtri a vuoto, lavato in acqua, rifiltrato e scaricato a mano in una pressa dove viene asciugato; previa macinazione è portato in forni di essiccamento e quindi raccolto in sacchi di juta. Questa seconda fase della lavorazione non espone ad inalazioni di vapori di acido acetico.

Nello stesso locale avviene un'operazione che, pur non essendo continua, è fonte di dispersione di grandi quantità di acido acetico: si tratta della filtrazione del triacetato di cellulosa, usato per particolari pellicole fotografiche; l'operazione si effettua in due filtri-pressa situati al piano terreno del fabbricato. Durante il cambio delle tele del filtro, che dura circa 30 minuti (10 per la svestitura del filtro, e 20 per la vestitura), dalle tele sfuggono notevoli quantità di vapori di acido acetico, solo in minima parte captate dall'aspiratore, della portata di 75 mc al minuto, situato sotto il filtro-pressa. Per di più, gli operai addetti al cambio maneggiano a mano nuda le tele da sostituire.

Gli operai sono forniti di occhiali e mascherina a filtro di cotone; durante il lavoro essi tengono vicino una bacinella contenente una soluzione di bicarbonato nella quale si lavano spesso le mani.

Per determinare la concentrazione dell'acido acetico nell'aria abbiamo eseguito 6 prelievi: i primi tre durante le condizioni normali di lavoro, gli altri durante le operazioni che disperdono l'acido acetico nell'ambiente; i risultati sono esposti nella tabella 1.

TAB. 1 - *Concentrazioni di acido acetico nell'atmosfera di un reparto di produzione dell'acetato di cellulosa.*

N.	Posizione del prelievo	mg/l	p.p.m.
1	fra due reattori durante un carico di acido acetico	0,183	75
2	fra due reattori durante la reazione	0,200	80
3	fra due reattori al piano di scarico	0,066	26
4	scarico dell'acetato di cellulosa	0,318	139
5	svestitura del filtro del triacetato di cellulosa	0,650	260
6	carico di acido acetico glaciale e al 60%, durante la determinazione della viscosità.	0,232	92

L'acido acetico, captato facendo gorgogliare l'aria in una soluzione di idrato di sodio, è stato determinato su tutti i campioni in due modi: 1) per titolazione dell'idrato sodico con acido solforico N/100; 2) col metodo colorimetrico di Vogel al nitrato di lantanio.

In condizioni di lavorazione normale le concentrazioni dell'acido acetico sono da 3 a 8 volte superiori alla massima concentrazione tollerabile; durante

lo scarico dell'acetato dai reattori e la svestitura del filtro-pressa del triacetato di cellulosa le concentrazioni dell'acido acetico ascendono a valori superiori alla massima concentrazione tollerabile da 9 a 26 volte.

Abbiamo sottoposto a visita medica gli operai del reparto.

1) S.M. di anni 38, lavora da 7 anni all'acetilazione, e spesso è addetto anche al filtro-pressa. Accusa bruciori agli occhi, alla gola, alle mani, specialmente durante il lavoro al filtro: lamenta digestione difficile. Obbiettivamente presenta la cute alle palme delle mani ispessita, nerastra, solcata da numerose ragadi superficiali dolenti alla palpazione; le congiuntive e il faringe sono intensamente arrossati; sul torace si apprezza un respiro molto aspro con espirazione prolungata.

2) R.M. di anni 37, lavora da 7 anni all'acetilazione, e spesso è addetto anche al filtro-pressa. Accusa bruciore agli occhi ed alla gola durante il lavoro. Da 4-5 anni soffre di tosse e catarro, più intensi nel periodo invernale; da un paio di mesi ha avvertito inoltre pirosi e qualche dolore epigastrico. Obbiettivamente presenta la cute palmare delle mani molto secca, screpolata, nerastra, le mucose congiuntivali e faringee arrossate ed edematose, un respiro molto aspro sul torace con espirazione prolungata, qualche sibilo e rouce specie nelle zone basali, discreta dolenzia alla palpazione dell'epigastrio.

3) B.A. di anni 37, da 11 anni lavora all'acetilazione e quasi ogni giorno è addetto al filtro-pressa. Accusa sul lavoro bruciori agli occhi, al naso, ed alla gola, talora pirosi post-prandiale; da qualche mese soffre di tosse e catarro accompagnati da modica dispnea da sforzo. Obbiettivamente presenta le palme delle mani secche, nerastre, callose e screpolate; intensa congestione congiuntivale e faringea con tonsille ipertrofiche e criptiche; sul torace modesta iperfonesi basale con basi poco mobili, respiro aspro con espirazione prolungata e numerosi ronchi e sibili in- ed espiratori.

4) L.M. di anni 41; da 12 anni lavora alla acetilazione e talora anche al filtro-pressa. Accusa sul lavoro bruciori agli occhi e alla gola, ed afferma che eventuali piccole ferite cutanee si cicatrizzano lentamente. Obbiettivamente presenta la cute delle mani nerastra, secca, con calli da lavoro e iniziale retrazione dell'aponeurosi palmare; denti incisivi anneriti con qualche chiazzetta di erosione; intenso arrossamento ed ipertrofia dei noduli linfatici delle congiuntive e del faringe; sul torace respiro aspro diffuso; polso 69 ritmico, duro, press. arteriosa 155-100, segni di arteriosclerosi generalizzata.

5) P.L. di anni 45, da 12 anni lavora all'acetilazione e saltuariamente al filtro-pressa. Accusa bruciori agli occhi e alla gola, talora pirosi e stipsi; lamenta il ritardo della cicatrizzazione delle ferite. Obbiettivamente presenta la cute delle mani molto callosa e screpolata; qualche chiazza di erosione ai denti incisivi e canini; mucose congiuntivali e faringe intensamente arrossati; sul torace respiro molto aspro diffuso, discreta dolenzia alla palpazione dell'epigastrio.

Questi cinque operai lavorano da 7-12 anni in un ambiente in cui l'acido acetico è sempre presente in concentrazioni che arrivano a 8 volte tanto quella massima tollerabile, e saltuariamente durante il lavoro ai filtri-pressa o in occasione di fughe per rotture di valvole o di altre parti dell'impianto possono ascendere a valori dell'ordine di 20-30 volte tanto la massima tollerabile. Essi lamentano disturbi e presentano lesioni della cute, delle congiuntive, dell'orofaringe, delle vie aeree, dei denti, dell'apparato digerente. Le alterazioni più evidenti della cute riguardano le mani, alla faccia palmare; essa è intensamente annerita, secca, solcata da profonde e numerose ragadi, talora dolorose alla pressione; le ferite esposte all'azione dell'acido acetico facilmente si trasformano in piaghe ad evoluzione torpida, a fondo grigio-nerastro. Le congiuntive sono iperemiche, la secrezione lacrimale è abbondante, ma in nessun caso si sono osservate alterazioni corneali; ancor più iperemico è l'orofaringe, che oltre ad essere intensamente arrossato presenta spesso una imbibizione edematosa della sottomucosa, più evidente ai pilastri e al velo pendulo; non rara l'iper-

trofia dei follicoli sottomucosi, sino alla tipica faringite granulosa; sempre evidente una secrezione sierosa, in qualche caso sieromucosa. Anche sul torace sono costanti i rilievi patologici, ora sotto forma di bronchite cronica catarrale, ora di bronchite cronica asmatiforme, con iniziale enfisema polmonare in un caso. Ragioni tecniche non ci hanno permesso di eseguire il sondaggio gastrico nè l'esame radiologico del tubo digerente; due operai su cinque esaminati hanno accusato pirosi postprandiale, talora accompagnata da dolori epigastrici e stipsi, e dolenzia alla palpazione epigastrica. I denti sono talvolta anneriti con qualche piccola chiazza di erosione alla superficie vestibolare degli incisivi e dei canini.

Come trattamento delle lesioni congiuntivali e faringee consigliammo il lavaggio oculare frequente e le inalazioni con soluzioni di bicarbonato, per le bronchiti l'uso di aerosoli alcalini, di broncodilatatori, ed altri rimedi sintomatici, nelle gastriti gli antiacidi ed una dieta adeguata, creme eutrofiche per la cute e, nei casi di ipercheratosi, unguenti cheratolitici. Ai fini della prevenzione suggerimmo per la cute l'adozione di creme barriera acidoresistenti, insistendo sulla necessità di provvedimenti tecnici per la protezione dell'organismo contro le concentrazioni ambientali troppo elevate di acido acetico: consigliammo cioè una più accurata manutenzione e una più sollecita opera di riparazione in caso di fughe, il rafforzamento degli impianti di aspirazione, la chiusura in apposito locale del filtro del triacetato di cellulosa, l'aerosolizzazione di soluzioni di bicarbonato durante le operazioni di vestitura e svestitura del filtro; l'uso di maschere a filtro di carbone o ad assorbimento alcalino potrà essere preso in considerazione solo come provvedimento momentaneo per circostanze eccezionali.

I nostri rilievi ci permettono anche alcune considerazioni di interesse più generale. È evidente che, nell'esposizione continuata, concentrazioni elevate di acido acetico sono sopportate discretamente dal punto di vista soggettivo: per quanto in certe operazioni si raggiungessero concentrazioni ben 26 volte superiori alla massima tollerabile, gli operai potevano continuare il loro lavoro senza molto disagio; d'altra parte confrontando il loro stato soggettivo con i disturbi che noi stessi provammo durante il sopralluogo nel reparto, riteniamo di poter fondatamente supporre che nella esposizione continuata all'acido acetico si stabilisca una certa assuefazione. Dal punto di vista obbiettivo l'azione protratta dell'acido acetico a concentrazioni assai superiori alla massima tollerabile è risultata dannosa e a lungo andare capace di provocare, sopratutto attraverso lesioni irreversibili delle vie aeree, una diminuzione permanente della validità lavorativa. L'attuale valore di massima concentrazione tollerabile appare stabilito con notevole cautela e probabilmente suscettibile di essere alquanto superato senza che ne debba derivare un danno apprezzabile per la salute degli operai. Non vi sono particolarità cliniche nè esami di laboratorio che permettano di stabilire la natura professionale delle singole manifestazioni descritte, perciò a questo riguardo ci si dovrà basare: 1) sull'accertamento di concentrazioni ambientali di acido acetico assai superiori alla massima tollerabile e continue, 2) alla contemporanea presenza delle lesioni nei vari apparati sensibili alla azione dell'acido. Infine è indispensabile che si proceda alla visita di assunzione degli operai destinati a lavorare in presenza dell'acido acetico, allo scopo di non esporre a questo rischio professionale coloro che sono affetti da bronchiti croniche e lesioni di una certa gravità dell'apparato respiratorio, da congiuntiviti croniche, faringotonsilliti croniche, gastroduodeniti e ulceri gastroduodenali in atto o pregresse, malattie della pelle; sarà anche opportuna la visita periodica almeno una volta l'anno.

RIASSUNTO

Sono stati esaminati 5 operai esposti da 7-12 anni a concentrazioni ambientali di acido acetico sino a 0,200 mg/l, cioè 8 volte maggiori della massima concentrazione tollerabile in condizioni di lavoro normali, e sino a 0,650 mg/l (26 volte maggiori) durante fasi particolari della lavorazione. La cute delle mani era intensamente annerita, solcata da ragadi talora dolenti, ipercheratosica, le congiuntive erano iperemiche, non si sono mai osservate lesioni corneali, il faringe era notevolmente congesto con edema e secrezione sierosa; sul torace si osservò un reperto di bronchite cronica, in 3 casi asmatiforme, in un caso con enfisema iniziale; i denti erano anneriti e presentavano chiazzette di erosione alla superficie vestibolare degli incisivi e dei canini; frequenti pure i disturbi riferibili a gastrite iperacida. È probabile che nella esposizione continuata si stabilisca una assuefazione verso il disagio soggettivo determinato dall'acido acetico, mentre le lesioni organiche a lungo andare possono essere tali da provocare una diminuzione permanente della validità lavorativa.

SUMMARY

PARMEGGIANI L., SASSI C. — *On the injuries to health caused by acetic acid in the production of cellulose acetate.*

Five workers who had been exposed for 7-12 years to atmospheric concentrations of acetic acid up to 0.200 mg/l, i. e. eight times higher than the maximum allowable concentration in normal working conditions, and 0.650 mg/l (26 times greater) during particular working phases, were submitted to medical examination: the findings were: the skin of the hands was considerably blackened, hyperkeratosic, furrowed with painful ragades, the conjunctivas were hyperaemic, no corneal lesions were observed, the pharynx was remarkably congested with edema and serous secretion. The examination of the chest revealed: chronic bronchitis, asthmatic-like in 3 cases, initial emphysema in one; the teeth were blackened and showed spots of erosion on the vestibular surface of the incisors and canines; also frequent the disturbances attributable to hyperacid gastritis. It is probable that with continuous exposure the workers can become hardened to the subjective discomfort caused by the acetic acid, whilst the organic lesions may, with the passing of time, be such as to cause a permanent decrease of the working capacity.

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Adv. 4(3), 227-230, (1955)

Studies of Acetate Metabolism in the Hereditary Obesity-Diabetes Syndrome of Mice Utilizing C^{14} Acetate

By William Parson and K. R. Crispell

With the Technical Assistance of Julia Vincour

THE PATHOGENESIS of obesity is a subject of lively interest.

Obese mice of the hereditary obesity-diabetes syndrome have been studied extensively by Jean Mayer.[1] Gruenbaum and Mayer have presented studies indicating that the obese mice do not catabolize acetate at the same rate as the nonobese animals. They have concluded that "the partial block of acetate utilization and resultant increased lipogenic acc. considered to represent the primary biochemical lesion of the syndrome".[2] This is a bold and exciting concept which warrants verification. This study presents data which fail to confirm the above report.

Experimental

Obese mice of the hereditary obesity-diabetes syndrome and litter mates were purchased from the Jackson Memorial Laboratory, Bar Harbor, Maine. The mice were housed individually in air-conditioned quarters and fed Purina chow. The animals were approximately 2 months of age when the studies were begun. The studies were carried out over a three-month period. During the course of the experiment the nonobese mice weighed 18 to 26 gm. and averaged approximately 22 gm.; the obese mice weighed 35 to 69 gm. and averaged approximately 45 gm.

The mice were fasted approximately 16 hours and then given intraperitoneal injections with 1 ml. of carboxyl-labeled sodium acetate containing approximately 1 microcurie of C^{14} (Tracerlab, Inc.). Each mouse was placed immediately into a metabolic apparatus for collecting CO_2. The techniques used in studying $C^{14}O_2$ excretion in rats described by du Vigneaud and associates were adapted for mice.

Air was first pulled through soda lime, concentrated sodium hydroxide and concentrated sulfuric acid to remove any CO_2 and water from the air. The CO_2 free air was then pulled through the mouse chamber at a rate of 0.5 L. per minute by a vacuum pump. The pressure within the chamber was kept around 7 mm. below atmospheric pressure.

The CO_2 from the mouse was collected in towers containing 2.5 N NaOH. The towers were changed at the end of the first half-hour, second half-hour, second hour and third hour. The NaOH from each collection was made up to 500 ml. with boiled distilled water and analyzed for carbonate content and radioactivity.

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Table 1.—*Excretion of $C^{14}O_2$ After Administration of C^{14} Acetate*

Date (1954)	Mouse No.	Weight gm.	Collection Period								Cumulative Totals at 180'		
			0 to 30'		30' to 60'		60' to 120'		120' to 180'				
			$BaCO_3$ mg.	Counts	$BaCO_3$ mg.	Counts	$BaCO_3$ mg.	Counts	$BaCO_3$ mg.	Counts	$BaCO_3$ mg.	Counts	Counts per mg. $BaCO_3$
	Controls												
3/3	7	18	136	25,300	116	10,510	112	6,600	408	1,610	1,102	44,020	40.0
4/27	7	20	151	17,650	50	4,700	221	12,500	166	5,540	790	40,840	51.4
3/10	12	21	239	10,700	136	7,110	391	4,880	298	418	1,158	53,108	45.9
3/11	3	21	210	44,650	181	9,530	268	2,605	556	1,810	998	58,065	58.1
4/25	3	26	356	33,220	222	11,450	362	10,395	688	3,490	1,628	61,555	37.8
3/17	9	23	256	11,030	238	10,230	414	3,725	452	1,639	1,360	56,894	41.8
5/9	9	21	291	29,500	262	9,810	340	6,140	552	1,790	1,188	47,270	39.8
3/22	6	21	*	*	*	*	652	5,700	650	1,530	1,928	92,080	47.7
5/7	6	25	258	57,500	140	6,390	344	5,300	696	3,965	1,438	73,055	50.8
3/25	18	21	242	55,100	159	9,160	526	6,430	590	3,505	1,508	72,135	47.9
3/30	10	21	283	51,500	260	9,770	616	6,430	602	2,320	1,762	73,020	41.4
4/30	10	25	310	48,500	162	13,300	404	7,330	602	3,200	1,478	72,990	49.4
4/1	19	20	151	15,660	66	4,895	218	1,280	218	1,162	656	25,337	38.9
	Obese												
3/2	1	36	299	37,200	118	4,200	350	4,600	352	1,120	1,140	46,520	40.8
4/12	1	39	281	36,450	226	4,300	432	4,960	616	2,630	1,508	47,840	29.9
5/13	1	51	428	65,000	351	13,290	794	7,060	710	2,340	2,226	87,380	39.3
3/5	2	36	292	49,500	210	12,090	546	6,510	656	2,665	1,654	70,865	42.9
3/9	8	35	292	33,350	222	17,050	334	10,260	530	2,210	1,478	62,890	42.5
4/14	8	40	334	52,000	299	6,350	498	4,800	362	1,660	1,451	65,040	45.4
3/15	4	43	280	45,700	291	9,630	486	3,500	484	1,530	1,554	59,560	38.4
4/13	4	48	351	50,900	246	9,840	710	5,960	702	2,250	2,112	68,500	32.4
5/22	4	52	346	49,800	338	9,100	782	4,850	786	2,790	2,424	57,540	23.7
3/19	5	45	264	41,700	236	16,490	494	6,580	498	2,120	1,192	70,495	47.6
3/22	11	45	324	35,500	218	15,480	386	19,650	388	3,780	1,292	74,504	57.7
4/21	11	49	284	33,400	240	16,400	502	5,830	396	1,640	1,232	67,870	52.5
5/24	11	53	274	31,100	290	15,700	540	6,830	558	2,370	1,652	58,960	36.1
3/29	13	43	316	42,500	256	21,850	560	12,350	528	1,940	1,660	78,290	47.2
4/22	13	55	346	50,400	306	25,700	656	20,250	622	3,370	2,080	99,720	43.1
5/27	13	60	520	61,600	198	5,250	764	5,300	856	2,060	2,738	87,240	31.9

* In mouse 6, $BaCO_3$ and counts were determined for first 60 minutes as a single period; values were 626 mg. and 85,000 counts.

For carbonate analysis 25-ml. aliquots were heated to boiling in 50 ml. Erlenmeyer flasks stoppered with CO_2 absorbing tubes. Two milliliters of 1 M $BaCl_2$ was added rapidly to precipitate barium carbonate. The flasks were heated in a boiling water bath for 20 minutes and allowed to cool before filtering through fritted glass crucibles of fine porosity. The precipitates were washed twice with boiled distilled water and once with absolute ethyl alcohol, care being taken to keep precipitates wet during filtration. Crucibles were dried under infra-red lamps and cooled in a desiccator before weighing.

For counting purposes: The procedure was the same as for analytical purposes except that the barium carbonate was not transferred to the fritted glass crucibles but was dried and pounded into aluminum counting disks with a metal instru-

ment which exactly fit into the disks. $BaCO_3$ was counted at maximum thickness in a Q gas windowless flow counter used with a Tracerlab scaler.

Sixteen studies were done on seven obese mice at different weight levels, and 16 studies were done on eight nonobese mice at different weight levels.

Results

The results are tabulated in Table 1.

Figure 1, showing the relationship between body weight and total CO_2 excreted (measured as $BaCO_3$) during the three hours, indicates that the obese mice excreted more CO_2.

Figure 2, showing the relationship between body weight and the specific activity of the excreted CO_2, indicates that there was some tendency for the obese mice to excrete CO_2 of lower specific activity.

Figure 3, showing the relationship between body weight and total radioactivity excreted in CO_2 during the three hours, indicates that the obese mice catabolized the injected acetate as well as the nonobese mice.

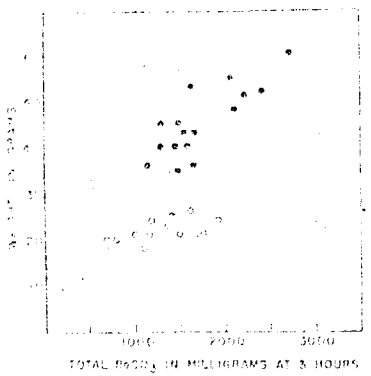

Fig. 1

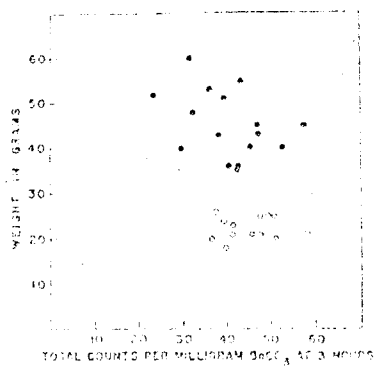

Fig. 2

Fig. 1. Relationship between body weight and total CO_2 excreted (measured as $BaCO_3$).
Fig. 2. Relationship between body weight and the specific activity of the excreted CO_2.

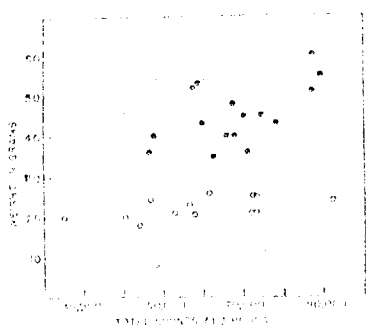

Fig. 3. Relationship between body weight and total radioactivity excreted in CO_2.

Discussion and Summary

This study fails to verify the report of a partial block of acetate utilization in the hereditary obesity-diabetes syndrome in mice. Inasmuch as Guggenheim and Mayer have not presented their studies in detail, the sources of the discrepancy between their results and ours is not apparent.

Acknowledgment

We would like to thank Dr. Robert B. Cahagan for his help in setting up this study.

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THE EFFECT OF ACETIC ACID ON THE STABILITY OF SERUM PROTEINS*

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(Received for publication, December 27, 1948)

Several investigators (1, 2) have shown that when horse serum is denatured a change in the relative distribution of electrophoretic components occurs. Van der Scheer, Wyckoff, and Clarke (1) found that heating of serum at 65° resulted in the formation of a single component, with the disappearance of the albumin and globulin peaks of the serum. This was confirmed by Davis, Hollaender, and Greenstein (2) who also demonstrated a similar phenomenon produced by ultraviolet radiation.

In the present study the stability of serum and certain plasma protein fractions at acid pH values in the presence of acetate has been investigated. To determine the stability of these proteins, electrophoretic analysis was carried out after removal of the acetate ions and the results are compared with those obtained with undenatured human serum.

EXPERIMENTAL

Usually three or four different samples of normal human serum were pooled and adjusted to the desired pH by dropwise addition of acetic acid with sufficient stirring to minimize any possible effects of local excess of acid. After standing at room temperature for 30 minutes, the solutions were dialyzed at 3–5° against 2000 cc. of 0.15 M sodium chloride for 2 days with several changes of the dialysate. The samples were then diluted with 0.1 N sodium diethyl barbiturate buffer of pH 8.6 to a final protein concentration of 1.87 per cent, *i.e.* 3.0 mg. of protein nitrogen per cc., and dialyzed in the cold for 3 days against liberal portions of this buffer.

The electrophoretic experiments were performed at 1.5° in a Tiselius apparatus (3) equipped with the schlieren scanning device of Longsworth (4). The concentrations of the electrophoretically separable components were estimated by the procedure of Tiselius and Kabat (5). The results are expressed as the ratio of the area of each component to the total area, ex-

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clusive of the δ- and ε-boundaries. Average values of the descending and ascending boundaries are given. Mobilities were computed from the descending patterns and refer to 0°.

Results

In Fig. 1 are superimposed the tracings of the electrophoretic patterns of normal human serum before and after exposure to acetic acid at pH 3.0 for 30 minutes. Electrophoresis of the acetic acid-treated serum showed a progressive increase in homogeneity of the material with the formation of a new peak. This new component, designated as D (for denatured), migrates with a mobility intermediate to mobilities of the α_2- and β-globulins.

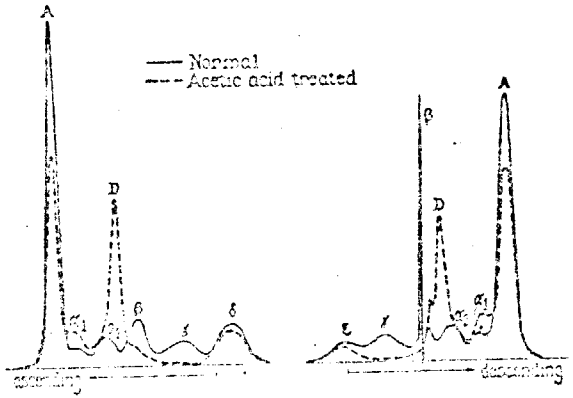

FIG. 1. Superimposed tracings of a 1.87 per cent solution of normal human serum before and after treatment with acetic acid at pH 3.0. Electrophoresis was carried out in a sodium diethyl barbiturate buffer at pH 8.6 and 0.1 ionic strength at a potential gradient of 5.3 volts per cm. for 12,600 seconds.

Exposure to acetic acid at pH 3.0 for 30 minutes was found to be necessary for the appearance of this component. In samples treated for 30 minutes, the D component has the same mobility as that of a sample treated for 48 hours, although in the latter case the D component is present in a somewhat higher concentration.

Effect of pH—The striking differences of the two patterns of Fig. 1 made it desirable to test the effects of acetic acid at various pH values. Table I shows the apparent distribution of the protein components observed in such experiments in which the pH was varied between 4.0 and 2.8. After exposure to pH 4.0, no significant changes were found. At pH 3.7, the α_1-globulin increased at the expense of the albumin. pH 3.4 causes a further shift in the concentration of these two components, and the D component appears. At pH 3.1, the amount of γ-globulin is reduced. These results indicate clearly that in the case of normal human serum in an acetic acid medium pH 4.0 represents the critical pH for the stability of the proteins. At pH values below 4.0 denaturation occurs.

In a series of experiments in which hydrochloric acid, lactic acid, or equilibration with a glycine-hydrochloric acid buffer was used for adjustment to pH 3.0, the formation of the D component was not observed. In these cases all the electrophoretic components of normal serum were present. However, a slight shift in the distribution of the relative concentrations of components was noticeable which was most marked in the case of the lactic acid-containing medium.

Stability of Plasma Fractions—Since it was found that the serum globulins are more specifically affected by the presence of acetic acid, the stability of plasma fractions was investigated. Various preparations were

TABLE I

Electrophoretic Distribution of Proteins in 1.87 Per Cent Solution of Normal Human Serum after Exposure to Acetic Acid at Various pH Values for 30 Minutes

Pretreatment of serum		Concentration in per cent as						Mobilities × 10^{-5} cm.² volt⁻¹ sec.⁻¹					
Concentration of acetic acid	pH	Albumin	Globulins					Albumin	Globulins				
			α_1	α_2	D	β	γ		α_1	α_2	D	β	γ
N		58.3	5.2	10.4		14.0	12.1	−6.4	−5.2	−4.1		−3.0	−1.3
0.264	4.03	57.1	5.6	12.1		13.2	12.0	−6.4	−5.2	−4.1		−3.0	−1.2
0.735	3.70	52.1	9.4	14.8		11.0	11.7	−6.3	−5.1	−4.0		−3.1	−1.3
1.47	3.40	47.0	14.9		25.0		16.1	−6.3	−5.2		−3.7		−1.2
2.94	3.10	45.0	16.0		34.8		4.2	−6.3	−5.3		−3.0		−1.2
5.88	2.80	47.8	10.0		40.8		1.4	−6.3	−5.2		−3.0		−1.3

Electrophoresis was carried out in a sodium diethyl barbiturate buffer at pH 8.6 and 0.1 ionic strength.

tested.[1] Only in Fraction IV-1, which contains α_1-, α_2-, and β-globulins, traces of albumin and γ-globulin, and which is characterized by a lipide content of 15.0 per cent, was a change similar to the one in serum noticed. This suggests the lability of lipide-protein linkages as the cause of the D peak. Another observation seems worth recording. In Fig. 2 are shown the electrophoretic patterns of γ-globulin before and after treatment with acetic acid at pH 3.0 for 30 minutes. The average mobility, $u = -1.2 \times 10^{-5}$ cm.² sec.⁻¹ volt⁻¹, remains unchanged. The somewhat greater homo-

[1] The products of plasma fractionation employed in this work were developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard Medical School.

geneity of the pattern in Fig. 2, *B*, however, is accompanied by an increase of irreversibly denatured protein which, in the presence of neutral salt, precipitates in the neighborhood of the isoelectric pH of this protein. Moreover, it seems of interest to consider this result in connection with the one described above; namely, that exposure of purified γ-globulin to pH 3.0 in the presence of acetic acid does not alter the mean mobility of the protein

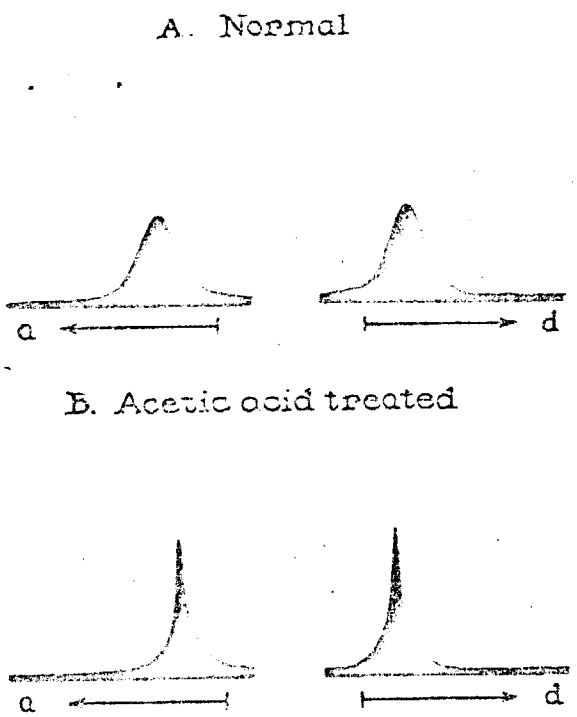

FIG. 2. Electrophoretic patterns of human γ-globulin. The patterns were obtained from a 1.0 per cent protein solution in sodium diethyl barbiturate buffer at pH 8.6 and 0.1 ionic strength at a potential gradient of 5.2 volts per cm. for 10,800 seconds.

fraction, whereas the same treatment causes this component to disappear in the serum.

DISCUSSION

It is apparent from the results presented in this paper that marked alterations occur in the distribution of electrophoretic components of serum after exposure to acetic acid for 30 minutes. These changes occur in a relatively short time and are dependent on the pH of the solution. No experiments to give a detailed account of the nature of this particular denaturation have been carried out. From the fact that exposure to acetic acid of the purified plasma fractions, *e.g.* albumin and γ-globulin, does not produce a significant shift of the electrophoretic mobilities, we may assume that the prevailing experimental conditions do not alter markedly the number of ionizable groups of these proteins. In the case of the lipide-rich protein fraction, Fraction IV, however, a change in the electrophoretic behavior was noticed. This leads us to believe that fission of lipide-protein linkages may be the cause of this phenomenon. The splitting of such bonds and a simultaneous recombination of molecules may readily yield aggregates of different size, but with a similar charge distribution such a mechanism could easily explain the formation of the D peak observed in the case of serum. A similar result was obtained by Tiselius and Horsfall in their work on the dissociation and reassociation of different hemocyanins (6).

From a comparison of the effect of acetic acid on serum proteins with that of other acids, *i.e.* hydrochloric acid, lactic acid, and the glycine-hydrochloric acid mixture, it is apparent that the formation of the D component at pH 3.0 is relatively specific for the presence of acetate ions. It therefore seems worth while to stress that denaturation does not depend on the nature of the protein and the acidity of the medium alone, but that the type of ions present in the medium also plays a rôle in determining the degree of stability of a protein. Moreover, it was noticed that under any given experimental condition the rate of denaturation may be enhanced considerably by the presence of neutral salts.

SUMMARY

Exposure of serum to acetic acid at pH values below 4.0 causes an irreversible denaturation of the proteins. This phenomenon is dependent on the pH of the solution. At pH 3.0, a new electrophoretic component, designated as D, was observed, which migrates with a mobility intermediate to mobilities of the α_2- and β-globulins.

The authors are indebted to Dr. J. L. Oncley of the Department of Physical Chemistry, Harvard Medical School, Boston, for supplying the plasma fractions.

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Acetate Incorporation into Lipids of Canine Lung Cells[1] (35995)

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Various type of cells are found in mammalian lungs (1). Of the cells found in the area of terminal air passages, type-II alveolar lining cells are currently thought to synthesize pulmonary surfactant. Said *et al.* (2), in 1966, reported that the alveolar cells lavaged from lungs of normal dogs had considerable metabolic activity consistent with a biosynthetic function. Alveolar cells (great alveolar cells, septal cells, corner cells, granulopneumocytes, or type-II cells) were tested for the presence of several enzymes in oxidative and synthetic pathways; they showed strong to moderately-strong reactions. They found that these large enzyme-rich alveolar cells were lacking in species with a deficiency of pulmonary surfactant. The correlation was made that the large alveolar cells were responsible for surfactant production. Autoradiographs of lung tissues from rabbits after an injection of tritium-labeled acetate showed radioactivity predominantly localized in the giant alveolar cells, as well as in a few alveolar macrophages at 30 min after the injection (3). From autoradiographs and chemical analyses of the lipid fractions at various time intervals, Buckingham *et al.* (3) concluded that the cytoplasm of the giant alveolar cells represented the active sites of phospholipid synthesis and turnover in the lung. To date, no direct evidence has shown the exact site of synthesis of the surface-active material found in the alveoli. The present study was undertaken to investigate the lipid synthesizing ability of cells washed from the lungs of dogs by bronchopulmonary lavage (4). Sodium acetate-1-^{14}C was incubated with these cells and their incorporation of the label into lipids was observed.

Materials and Methods. Three beagle dogs (3.5 years old) from the Lovelace Foundation beagle dog colony were used. Dogs anesthetized with pentobarbital sodium were intubated with a double lumen Carlens catheter and the pulmonary lavage was accomplished by techniques previously documented (5). The lavage fluid was removed from one lung (the other lung sustained the dog), stored at 5° until all lavages were completed and the fluid thus obtained was centrifuged for 600 g/min. The supernatant fluid was discarded, the cellular button was resuspended in Hanks' balanced salt solution, and the cell concentration was determined in a Model F Coulter counter (~1.4 × 10⁶ cells/ml). Counting vials, thoroughly cleaned and sterilized, were used as the incubation containers. A 150 μl aliquot of Hanks' solution (containing 500 units of penicillin and 500 μg of streptomycin sulfate) was pipetted into each of six vials, followed by 250 μl of the cellular suspension and finally 50 μl of a sodium acetate-1-^{14}C solution (1.136 μCi and 52 mmoles) was added. The cellular reactions were allowed to take place in a Dubnoff metabolic incubator at 37°, with gentle shaking (100 strokes/min), and a vial was removed every 30 min. After incubation, the metabolic reaction was terminated by the addition of CH_3OH (the first step of the Bligh and Dyer (6) lipid extraction procedure). Addition of the proper proportions of $CHCl_3$ and H_2O allowed the separation of the $CHCl_3$ phase containing most of the lipid-like materials. Reextraction of the CH_3OH-H_2O phase yielded a second $CHCl_3$ extract that was combined with the first. The $CHCl_3$ fraction was evaporated to dryness under N_2 and the lipid residue was redissolved in 60 μl of $CHCl_3$.

Thin-layer (250 μm) chromatograms (2 ×

[1] Work performed under Contract No. AT(29-2)-1073 between the U.S. Atomic Energy Commission and the Lovelace Foundation for Medical Education and Research.

TABLE I. Lipids[1] Synthesized from NaOAc-^{14}C After Incubation with Lung Cells Derived from a Pulmonary Lavage.

Incubation time (min)	Percentage of total lipid								Percentage of ^{14}C incorporated into lipids
	CHOL	FFA	TG	SE	PI + PS + SPHI	PC	PC + PDME	PE	
30	13	32	21	5	2	16	5	2	3.1 ± 0.1
60	16	27	21	6	2	17	8	2	4.7 ± 0.2
90	23	24	15	9	1	15	8	2	5.2 ± 0.5
120	27	21	20	4	2	15	8	2	5.4 ± 0.4
150	33	21	17	3	1	13	8	2	5.9 ± 0.2
180	36	18	14	2	1	12	9	2	5.7 ± 0.2

[1] Other lipid solvent-soluble material contained a few percent of the ^{14}C radioactivity, but the components listed are the principal identifiable compounds. CHOL, cholesterol; FFA, free fatty acids; TG, triglycerides; SE, sterol esters; PI, phosphatidyl inositol; PS, phosphatidyl serine; SPHI, sphingomyelin; PC, phosphatidylcholine; PG, phosphatidyl glycerol; PDME, phosphatidyl N-dimethylethanolamine; PE, phosphatidylethanolamine.

20 cm) on silica gel G and HR were prepared and developed as previously described (4). Each sample from the lung cell-NaOAc incubation and mixtures of standard lipids were chromatographed on the same plate in four solvent systems, two neutral and two phospholipid resolving systems (4). The chromatograms were developed (13 cm), air dried, and exposed to I_2 vapor. The standard lane was marked to identify a particular area with a particular lipid. Zonal scan analysis (7) of the radioactivity distributed on thin layers was used to achieve high counting efficiencies and high resolution of the separated lipid components. Zones (2 mm wide) of silica gel along the chromatograms were scraped into counting vials. A liquid scintillation spectrometer with a toluene naphthalene methylcellosolve water cocktail was used to assay for carbon-14 at a counting efficiency of approximately 46%. The total activity recovered from each plate was compared to the activity recorded from a direct pipetting of an aliquot of the sample into a counting vial which was used as the standard. This provided a check on quenching, instrument, and technical errors involved in the procedures. The total activity in each lipid class was compared to the total activity on the plate and the percentages of the activity were calculated. The values are averages from three incubation reactions at each time period and from four TLC plates from each $CHCl_3$ extract.

Results and Discussion. The data in Table I show that the cells obtained from a lung lavage can actively synthesize phospholipids and neutral lipids from acetate. The cells obtained from the lungs of beagle dogs by lavage and identified by their morphologic appearance represented a variety of cell types including alveolar cells, macrophages, lymphocytes, neutrophils, and bronchial epithelial cells (4). Although maximal incorporation of ^{14}C into lipids occurred at 2.5 hr of incubation, active cellular metabolism was still evident after 3 hr of incubation (5 hr after removal from the lung). Unfortunately, the techniques do not permit the identification of the cell type or types responsible for lipid synthesis. It is quite possible that cell death occurred during the 5 hr time period and that only a select group of cells were the active participants. Penicillin and streptomycin were used in the incubation media to prevent the development of bacteria, which could have contributed to total acetate utilization. However, in other studies in this laboratory, lung lining cells cultured in Eagle's basal medium containing 10% serum and antibiotics did not show a pH shift (color change) until 96 hr postlavage. At this point Gram stains revealed bacterial overgrowth.

One of the principle routes for acetate incorporation into lung lipids is in the synthesis of fatty acid, which quickly become esterified and form triglycerides and phospholipids (8, 9). The data in Table I show that

after a 30-min incubation most of the total lipid ^{14}C was in the free fatty acid fraction (32% of total lipid ^{14}C). This is a greater incorporation of acetate into lipids than has been reported with lung slices (9). Phosphatidyl choline (PC) contained the highest amount of ^{14}C among the phospholipids. This was not unexpected since PC, allegedly the most active component of pulmonary surfactant, has been shown (4) to be the predominant phosphatide in both pulmonary surfactant and alveolar lining cells.

The amount of ^{14}C, however, appeared to increase throughout the time of incubation in the phosphatidylglycerol, dimethylethanolamine and cholesterol fractions. The increase in ^{14}C in these phospholipids probably indicates that the component(s) are synthesized at a relatively slow rate and that they have not reached an equilibrium concentration. Also, with ^{14}C-labeled fatty acids in the system, a net increase in ^{14}C due to esterification would be entirely possible. A linear increase in cholesterol-^{14}C for 5 hr was observed and was probably related to a net synthesis from ^{14}C-acetate. Cholesterol, a necessary component of biological membranes, is associated with both both alveolar lining cells and pulmonary surfactant (4), the membranous layer at the air tissue interface within the alveoli.

The amount of ^{14}C in other lipid components (TG, SE, PC) appeared to increase and then decrease with time of incubation (Table I). This is probably a real phenomenon, since most of the ^{14}C would be in the fatty acids which, with time, would be synthesized, esterified, and then catabolized to acetate (beta oxidation).

Although Buckingham *et al.* (3) demonstrated the uptake of ^{14}C-acetate into palmitate in the "special" alveolar cells *in vivo*, their studies did not rule out the possibility of extrapulmonary synthesis. Chida and Adams (10) incubated acetate-1-^{14}C with lung slices and showed the radiolabel incorporated predominantly into lecithin, diglycerides, and triglycerides with some ^{14}C in cholesterol and cholesterol esters. These studies (3, 10), although useful in showing the active metabolism of lung tissue, do not specifically address the question of the site of synthesis of the pulmonary surfactant lipids.

This work demonstrated that the lung cells removed by pulmonary lavage and principally derived from that region where surfactant is found were metabolically active in incorporating acetate into lipid components such as those found in pulmonary surfactant. Since these cells are able to synthesize the major lipid components of pulmonary surfactant, they may be considered a some possible site of the synthesis of the surface-active material.

Summary. Cells principally derived from the alveolar portion of the lungs of beagle dogs were obtained by pulmonary lavage and incubated with ^{14}C-acetate. Labeled lipids were isolated and quantitated by usual procedures (thin-layer chromatography, scanning, and liquid scintillation counting). The cells actively incorporated the acetate into both neutral lipids and phospholipids. Phosphatidylcholine was the predominantly labeled phosphatide and cholesterol was the predominantly labeled neutral lipid. The results show that the alveolar lining cells may be considered as a site of the synthesis of the lipid components of pulmonary surfactant.

The authors are indebted to B. A. Mazzocchi for assistance in the [illegible] Mrs. A. Fouts for help with the incubations, Mr. J. B. Nash for running the Coulter counter and making cell counts, and Drs. K. J. Blackburn, J. R. Hamilton, R. K. Jones, R. B. Lindsay, R. C. McCoy, and J. V. Pierce for critical review of the manuscript.

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ACETIC ACID IN THE TREATMENT OF SUPERFICIAL WOUNDS INFECTED BY PSEUDOMONAS AERUGINOSA

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Summary Patients with burns or superficial wounds infected by *Pseudomonas aeruginosa* were allocated at random to treatment with dressings soaked with acetic acid (ten patients) or with chlorhexidine or hypochlorite (ten patients). Lesions were swabbed once a day for seven days, and after overnight incubation growth was scored as none (0) to very heavy (5). In ten patients treated with acetic acid the mean growth score fell from 4·1 on day 0 to 0·7 on day 7; in the control group the score remained much the same (3·0–3·8) throughout the trial period. In only one of the acetic-acid-treated patients was therapy completely unsuccessful.

Introduction

ATTEMPTS to prevent the colonisation of superficial wounds and, particularly, burns by *Pseudomonas aeruginosa* have met with limited success. Silver nitrate seems to be the best of a number of topical agents (Caron et al. 1966). However, when prophylactic measures have failed or have not been applied, the problem of eradication remains; topical polymyxin has proved most useful in such cases, although in a substantial minority the organism is not removed (Lowbury 1966).

Vinegar has long been included among the agents used in the treatment of superficial wounds—Hippocrates recommended it, among other medicaments, for the treatment of ulcers (Adams 1939). Taylor (1916) found that acetic acid had particular value in eradicating *Ps. aeruginosa* from superficial wounds, but Pulvertaft (1943) was unable to confirm this. However, *Pseudomonas* infections of other sites such as the ear (Smith 1924, Ochs 1950), chest (Currence 1952), and urinary tract (Kass and Sossens 1959) have been successfully treated with acetic acid. These clinical observations were supported by Owen (1946) who showed that acetic acid in vitro was notably more inhibitory to gram-negative than to gram-positive organisms.

In the New Mulago Hospital, Kampala, it has been the practice of some surgeons to treat *Ps. aeruginosa* infections of superficial wounds and burns with acetic acid. Clinical results seem favourable. This trial was undertaken to try to obtain more accurate information on the bacteriological and clinical effectiveness of such treatment.

Clinical Material and Methods

Patients

Patients were considered for inclusion in the trial whenever *Ps. aeruginosa* was isolated in the routine examination of pus from superficial wounds or burns. If permission was given by the clinician in charge, the patient was allocated randomly to the treatment or control group by selection from a set of twenty sealed envelopes. Ten envelopes contained instructions to treat the patient with acetic acid, and ten contained instructions not to treat with acetic acid.

The antibiotic therapy before and during the trial was noted and clinical progress was assessed in consultation with the clinician. Because of the odour of acetic acid, a blind assessment was not feasible.

The ten patients in the treated group were given twice-daily dressings with 5% acetic acid and the ten controls were given twice-daily dressings with chlorhexidine ('Hibitane') or hypochlorite ('Eusol'). Wounds were swabbed with gauze soaked in the same solution. Treatment was continued for 7 days unless the patient's clinical course necessitated interruption.

Bacteriology

Absorbent cotton-wool swabs were used to sample the bacterial flora of the lesion each morning, before the first dressing of the day. Swabbing was done in a uniform manner by one of us (A. Z. L.) whenever possible, but at other times by a nurse acting under his instructions. Samples were examined on each of the seven days of the trial, with a few unavoidable lapses, and in some cases for a variable period after the trial.

Human-blood-agar and MacConkey-agar plates were inoculated in a standard manner and examined after overnight aerobic incubation at 37° C by two of us (I. P. and R. F.) who did not know which treatment schedule had been used. *Ps. aeruginosa* was identified by tests previously described (Phillips 1967) and other organisms were identified by conventional methods. The amount of growth was roughly indicated from none, labelled 0, to very heavy, labelled 5. All strains of *Ps. aeruginosa* isolated were pyocine typed, by the method of Gillies and Govan (1966), using their basic set of eight indicator strains, plus a further five to subdivide pyocine type 1.

Tests for carry-over of acetic acid were performed by inoculating swabs from patients across thin lawns of *Ps. aeruginosa* on blood-agar plates.

Results

Eight patients in the group treated with acetic acid, and seven in the control group were men. Apart from a newborn infant and a girl aged 6, both controls, all were adults.

The infected lesions included bedsores (seven), skin lacerations (six), and the site of an inguinal block dissection, a chest burn, a Kaposi sarcoma, balanoposthitis, Buruli ulcer, diabetic gangrene, and an ulcerated cephalhematoma (one case each).

All the patients had had courses of two or more antibiotics, except one who had penicillin only. Similarly, all had had topical treatment with chlorhexidine or hypochlorite, or both.

The table shows the results of daily bacteriological sampling of the wounds of treated and control groups, and the mean daily results for each group. In seven out of ten cases, twice-daily dressing with acetic acid eliminated the organism by the seventh day, whereas dressings with chlorhexidine or hypochlorite had a similar effect in only one out of ten cases. In the treated group 30 out of

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GROWTH OF *PS. AERUGINOSA* IN SWABS TAKEN FROM PATIENTS TREATED WITH ACETIC ACID OR WITH HYPOCHLORITE OR CHLORHEXIDINE

Patient	Growth score in acetic acid group on day: 0	1	2	3	4	5	6	7	Patient	Growth score in hypochlorite/chlorhexidine group on day: 0	1	2	3	4	5	6	7
1	4	4	3	3	2	0	0	0	3	4	4	4	..	4	4	4	4
2	4	3	0	0	0	0	0	0	5	4	4	4	4	5	4	4	3
4	4	1	0	0	0	0	0	0	8	5	4	.	4	4	4	4	5
6	4	0	0	0	0	4	0	2	10	4	3	..	..	..	..	..	..
7	4	1	1	.	4	0	4	0	11	3	4	5	4	3	..	..	..
9	2	1	0	0	0	0	0	0	12	5	4	4	3	4	3	4	5
15	5	5	4	3	0	0	0	0	13	2	1	0	0	2	1	2	1
17	5	4	4	4	4	4	4	4	14	4	..	5	5	5	4	5	5
18	5	0	1	4	0	1	0	0	16	2	3	3	3	3	0	0	0
19	4	3	2	0	0	0	.	..	20	3	4	4	4	4	4	4	4
Mean	4·1	2·2	1·5	1·2	1·0	0·5	0·6	0·7	*Mean*	3·3	3·1	3·6	3·4	3·8	3·0	3·4	3·4

77 swabs had no *Ps. aeruginosa*, but only 5 out of 68 in the control group were free from the organism.

In three instances the trial was curtailed, because of skin grafting (cases 10 and 11) or because the patient was discharged (case 19). In four instances one of the daily swabs was inadvertently not taken (cases 3, 6, 12, and 18).

In six treated cases, cultures were continued after acetic acid had been stopped. In case 1 *Ps. aeruginosa* returned in large numbers, but in the remainder either the wounds remained free (cases 4, 6, and 15) or there was transient light contamination only (cases 2 and 7, in which swabs were continued for 14 and 12 days, respectively, and *Ps. aeruginosa* was isolated in very small amounts on 4 and 2 days respectively). Four cases did not have follow-up cultures either because they were discharged immediately (cases 9, 18, and 19) or because there had been no response to acetic acid (case 17).

Tests for carry-over of acetic acid were difficult to interpret because of the presence of many other organisms, but did not suggest that the phenomenon was important.

There was clinical improvement in eight of the treated patients and no change in two (cases 6 and 17). Skin grafting was performed successfully on five patients. There was no clinical change in patients in the control group, although two had minimal signs of infection throughout (cases 11 and 16). Four control patients were subsequently improved bacteriologically and clinically by acetic acid, although another (case 16) did not respond.

Most of the patients found the stinging of the application immediately unpleasant, but the pain did not continue. In only one patient, the child of 6, were complaints so vociferous as to make us consider stopping treatment.

Acetic acid had no effect on organisms other than *Ps. aeruginosa*. Including controls later treated with acetic acid, the following organisms either replaced *Ps. aeruginosa* or were present throughout: *Staphylococcus aureus* in twelve cases, *Proteus* in nine, *Streptococcus faecalis* in five, *Escherichia coli* in four, and *Str. pyogenes* in one.

The following pyocine-typing patterns were seen: 1·3 (five cases), 1·5 (five cases), 1·2 (four cases), 10 (four cases), 1·4 (two cases), and 3 (one case). Two patients had two strains in initial and final swabs. The strains isolated from two patients were accidentally not typed. In all cases strains isolated at the end of the period of study had the same pyocine-typing patterns as those found at the beginning, both where *Ps. aeruginosa* persisted throughout, and where it reappeared after treatment.

Discussion

These results suggest that acetic acid is effective in the eradication of *Ps. aeruginosa* from superficial wounds. In eight out of ten cases, twice-daily dressings eliminated the organism in less than a week, and the wounds remained free from *Pseudomonas* in most of the cases in which follow-up cultures were possible. These results seem to be as good as those obtained by the use of topical polymyxin on burns (Lowbury 1966) although it should be noted that most of the lesions in our series were much smaller than burns requiring admission to hospital.

Success was not complete. In one patient with an infected Kaposi sarcoma the organism persisted undiminished in quantity throughout, and in another with bedsores, it returned in large amounts as soon as treatment ceased. In two more it was present transiently in follow-up cultures but did not gain a foothold. In each of these cases pyocine typing suggested that the findings were due to persistance rather than reinfection. It seems probable that in the case of the Kaposi tumour, infection was too deep-seated to be reached by any topical treatment.

Although formal tests were not completely successful, several observations support our conclusion that carry-over of acetic acid to culture plates was not responsible for our findings. First, swabs were taken 15 hours after dressings, when the concentration of acetic acid was expected to be at its lowest. Second, the disappearance took place gradually, over a period of a few days, during which time the amount of acetic acid was presumably the same. Third, in one case the organism was cultured in large amounts throughout. And finally, follow-up cultures were generally free from *Ps. aeruginosa*.

The lack of effect of acetic acid on organisms other than *Ps. aeruginosa* was striking, and applied to both gram-positive and gram-negative species—especially *Staph. aureus* and *Proteus* which actually increased in quantity in many of the wounds.

In most instances the disappearance of *Ps. aeruginosa* was accompanied by clinical improvement of the wound and when skin grafting was necessary, it was successful in spite of the appearance of other organisms.

We conclude that acetic acid is a useful agent for the treatment of superficial wounds infected with *Ps. aeruginosa*. A disadvantage is that it can cause quite severe stinging, but this should be set against ready availability, cheapness, and bacteriological and clinical efficacy.

We thank the medical staff of Mulago Hospital for permission to study their patients; Dr. M. C. Pike and Mr. S. K. Lwanga for help in defining the statistical interpretation of the results; Dr. R. R. Gillies and Dr. J. R. W. Gibson for supplying pyocine typing strains for pyocine typing; and Prof. R. Blowers for helpful criticism of the manuscript. One of us (A. Z. L.) was in receipt of a grant from the Stockholm Students' Fellowship.

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References at foot of next column

SODIUM ACETATE

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ACTION ON THE STRIATED MUSCLE BY SOME SUBSTANCES THAT PRODUCE SYSTOLIC ARREST OF THE HEART

By J. Pisanty*

Introduction

In the present article, the effects on the skeletal muscle are studied, of a series of substances whose common property is the producing of a cardiac effect characterized by a progressive decrease in the relaxation of the tissue (fiber), that terminates with a systolic arrest of the hearts in cold-blooded animals. This study forms part of a program worked out by Doctor Arturo Rosenblueth for comparing the properties of the different types of muscular tissue and their responses to the various substances that exert an effect on them.

The systolic arrest of the heart in the frog was considered up to a few years ago as a specific reaction of the digitalic medications and their glucosides, of the excess of calcium ion and of the ionized salts of barium. The specific nature of this phenomenon has been in the process of being ~~diminished~~ weakened from day to day since Méndez y Montes (1943) and Krayer, Méndez and Moe (1944), demonstrated that various alkaloids obtained from some species of Veratrum are capable of producing systolic arrest of the ventricle of the frog and other effects that precede this terminal event.

According to recent observations by Méndez (1946), a group of substances exists that are related to each other by their chemical formation, but which can be grouped by their characteristic of being combined with the SH groups of the sulfhydralic enzymes, and that are capable also of producing systolic arrest of the heart in certain cold-blooded animals. This group of substances known by the general name of sulfhydralic reagents, have already been studied Hellermann, Chinard, and Deitz (1943) and by Guzman Barron and Singer (1945) in isolated enzymatic systems. Mendez (1946) initiated his research on living tissues.

There is undoubtedly considerable interest in knowing the behavior of the skeletal

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muscle vis-à-vis this group of substances whose common effect is to produce systolic arrest in the heartsof cold-blooded animals.

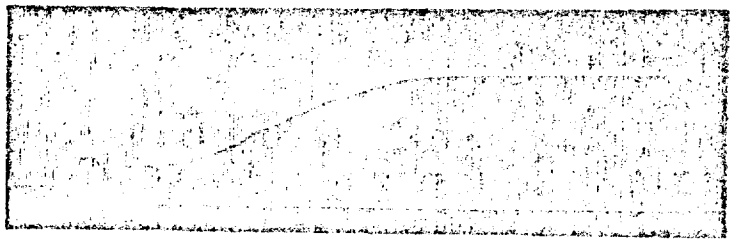

Fig. 1. Biceps of a turtle subject to stimulus every 30 seconds. At the moment of the signal, 0.5 mg. of ouabain was injected.

METHOD

In the results that we describe at length, analysis is made of the effects of the striated muscle of two cardiac glucosides: digitoxin (Nativelle digitalin) and ouabain, that can be considered as genuine representatives of the broad group of glucosides with "digitalic effect," as well as those of three of the specific reagents of the sulfhydralic enzymes: orto-iodosobenzoic acid, iodoacetamide, and hydrochloride of the 3-amino-4-hydroxybenzolarsenious acid.

First the sartorius muscle of the frog was tried as had been done by McKeen Catell (1938) in a study of the effect of strophanthin. In the same way the method used by del Pozo (1943) in his research on the effects of certain strophanthins on the circulated gastrocnemius of the cat was tried. It was not possible with ~~any~~ either of the two methods cited to obtain muscular shortening by means of the substances used so that an adequate preparation was sought for that would permit injecting the substances into a muscle whose contractions can be easily registered and where furthermore the injections are spread as little as possible to the rest of the animal, for which reason a muscle was required whose circulation is very slow, at the same time having an artery with sufficient light so that injections could be made without too many difficulties.

In a turtle whose brain was removed and without the upper part of the shell, both biceps are disected, the distal end of the latter muscles are tied to a sufficiently resistent wire (thread), and the latter is connected to the recording ~~isophorme~~ isotonic myograph. On the other hand, a point is driven into the scapulo-humeral articulation (joint) and is attached to a support.

The distal end of the biceps, already tied, is cut, leaving it thus attached to the animal only by its insertion into the shoulder, a site through which its nutrient vessels penetrate. The sub-clavian artery is disected. The brachial plexus is sought out below the

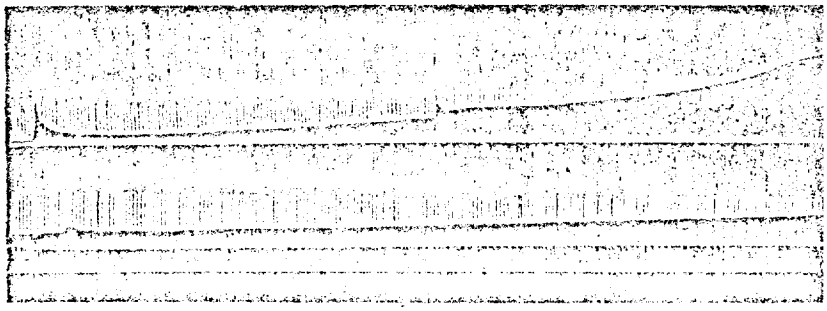

Fig. 2. Two biceps of a turtle. The one in the upper line (curve) was injected with 0.6 mg. of digitoxin, in divided doses. The lower line is the control without injection. Indirect stimuli of 0.05 microfaradays at 30 volts every 30 seconds. ~~T: 1 min.~~ Time : 1 min.

scapula, it is disected, its proximal end is crushed in order to avoid the arrival of nervous impluses to the muscles that it innervates, and electrodes are placed on it that will serve as a stimulus. The extremity is cut at approximately the shoulder joint (articulation) in order to avoid registering its movements that might interfere with those of the biceps.

With this preparation, it is possible to inject into the irrigated scapular muscles and into the biceps, without causing the animals death, quantities of the substances mentioned that are very much greater than those that it is possible to inject into mammals without obtaining an adequate effect.

The solutions of all the substances that are injected were previously adjusted to a pH of 7.5. Whenever it was possible, an osmotic concentration was injected with approximately the osmotic concentration of Ringer's fluid.

The concentration used for the inhibitors of the reagents of the sulfhydralic enzymes was always 5 to 10 times greater in weight than that of the reagent tried; e.g., for 10 mgs of iodoacetamide, 100 mgs of sodium acetate.

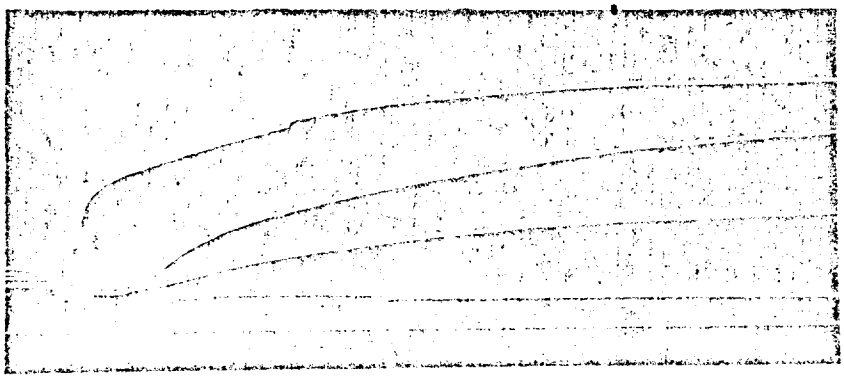

Fig. 3. Shortening of the biceps of four different turtles with iodoacetamide. The dosages used were 100, 20, 10, and 2 mgs. respectively for the muscles of the lines beginning with the upper line and so on. Previously it was arranged that the responses to a stimulus should be equal by modifying the lever arm.

RESULTS

A) Cardiac glucocides. a) Ouabain. With the injection of dosages beginning with 0.05 mg, there was obtained a shortening of the muscle, a shortening that is irreversible, progressive, and that reaches a level higher than the maximum contraction yielded by a single indirect shock. Smaller dosages did not produce shortening nor did they have any effect after the application of stimuli ~~through~~ to the nerve. When periodic stimulations were carried out, an effect was observed similar to the positive inotropic effect that is registered in the hearts of cold-blooded animals. The effect of 0.5 mg. of ouabain in a biceps of a turtle is illustrated in fig. 1.

b) Digitoxin. In essence, the effect of this substance is equal to that of ouabain. Because of the difficulties existing in dissolving it, it was usually injected in divided doses. In fig. 2 the effect is seen of 0.6 mg. of digitoxin injected in divided doses, in comparison with a muscle into which no injection was made, and an increase in the extent of the contractions is seen.

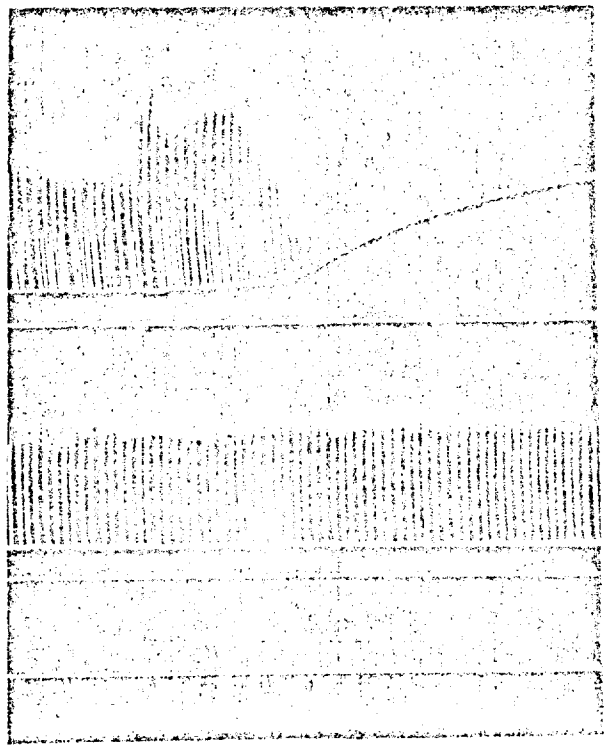

Fig. 4. Both gastrocnemii of a cat. Indirect stimuli every 30 seconds. In the upper register, 200 mg. of iodoacetamide. Note the positive inotropic effect. The lower register is for the control muscle which was not injected.

It is interesting that the shortening produced with these drugs was always the maximum and that it was not possible to obtain intermediate degrees of shortening by varying the dosage. Stimuli were not required to initiate this phenomenon.

c) Other substances. Both ouabain and digitoxin were injected along with glutathione, cysteine, glucose, sodium lactate, and sodium acetate without obtaining modifications of their effects. Neither was it possible to impede their action with atropine, which however could be done successfully under certain conditions in the smooth muscle (Alanis, 1947).

B) Sulfhydralic reagents. a) Iodoacetamide. This substance easily produces irreversible shortenings of the skeletal muscle preceded by a clear increase in the extent of the contractions. For the biceps of the turtle, dosages of 1 to 2 mg. are ineffective and dosages of 4 to 5 mg. indeed produced shortening, this being greater as the quantity injected is greater, as can be seen in fig. 3, with dosages of 2, 10, 20, and 100 mgs. This reagent is the only one with which a shortening could be reproduced in the gastrocnemius of the cat with a dosage of 200 mgs., with a very clear increase in the extent, as can be seen in fig. 4. Various trials, in the cat, made with smaller dosages, did not yield any results.

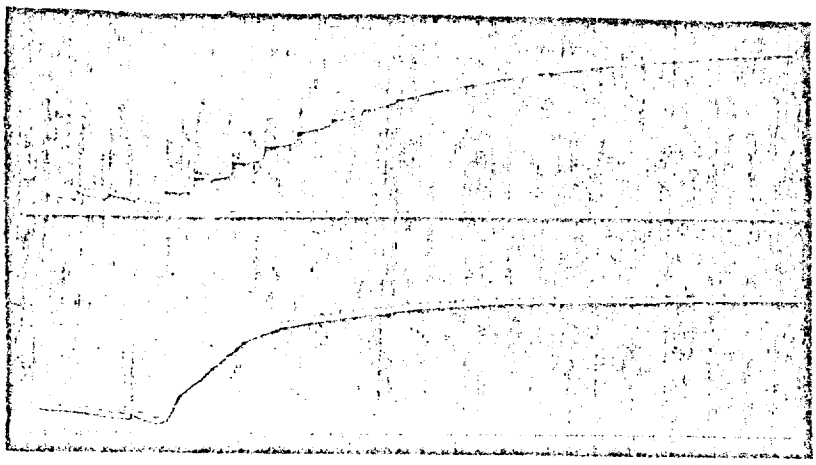

Fig. 5. Influence of the frequency of stimulation. There are two biceps of a turtle into which the same quantity of iodoacetamide was injected. Indirect stimuli are applied every 5 minutes to the upper line, and no stimulation is applied to the lower line.

Both iodoacetamide as well as the reagents studied in b) and in c) produce a spontaneous shortening, that is to say, that no stimuli are required to initiate it; furthermore it is not influenced by the frequency of stimulation. Thus, in fig. 5 the similar effect is seen of equal amounts of iodoacetamide, with and without stimulation.

b) O-iodosobenzoic acid. The minimum dosages necessary of this reagent are slightly higher than those for iodoacetamide and, like the latter, produce a more intense shortening as the dosage is increased, as is illustrated in fig. 6. Stimuli are not required to initiate its action, which is not reversible.

c) Hydrochloride of 3-amino-4-hydroxybenzolarsenious acid.* The minimum dosages of this reagent for obtaining a decrease in the length of the biceps of a turtle were from 120 to 150 mg. Fig. 7 is the record of the shortening produced by a dosage of 200 mg.

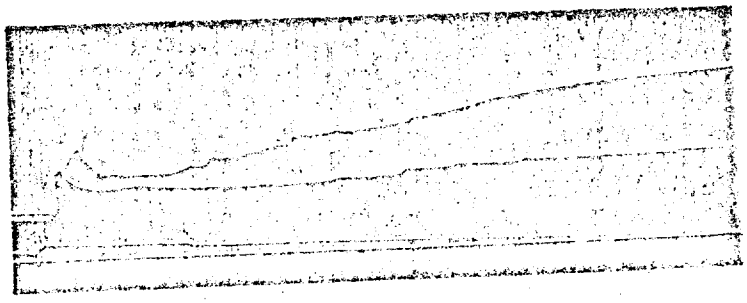

Fig. 6. Biceps of the turtle. The responses to a stimulus have been previously equalized by varying the length of the lever arm. The upper line is for a biceps injected with 20 mg. of o-iodosobenzoic acid, the intermediate line with 10 mgs., and the lower line with one.

This substance is the most difficult to study of all those that were used because of the large concentration in which it is required to be used and because of the difficulties inherent in its neutralization.

d) Inhibiting agents. The shortening produced by the sulfhydralic reagents is not reversed spontaneously, nor has it been possible to make it reversible with any of the inhibitors ~~with~~ of the sulfhydralic reagents studied. This shortening is nevertheless prevented by the addition of SH groups in the form of cysteine or of glutathione and by different substances related to the intermediate metabolism of glucose and by glucose itself as is seen in fig. 8.

Of these substances related to the intermediate metabolism of glucose, there were tried sodium lactate, sodium acetate, ferric citrate, and glycerin, all of which are capable of impeding the action of the sulfhydralic reagents.

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* This reagent, in anhydrous form, was generously supplied by the Park Davis & Company.

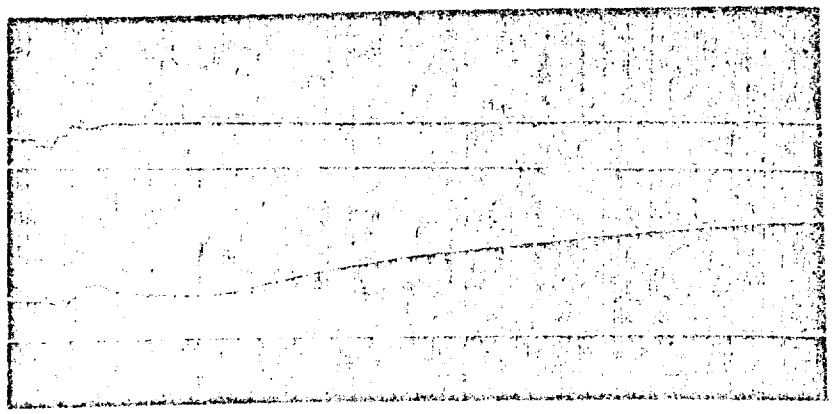

Fig. 7. Above: control muscle, not injected. Its line is parallel to the line of zeros. Below: a muscle into which was injected 200 mg. of hydrochloride of 3-amino-4-hydroxybencenoarsenious acid in neutral solution.

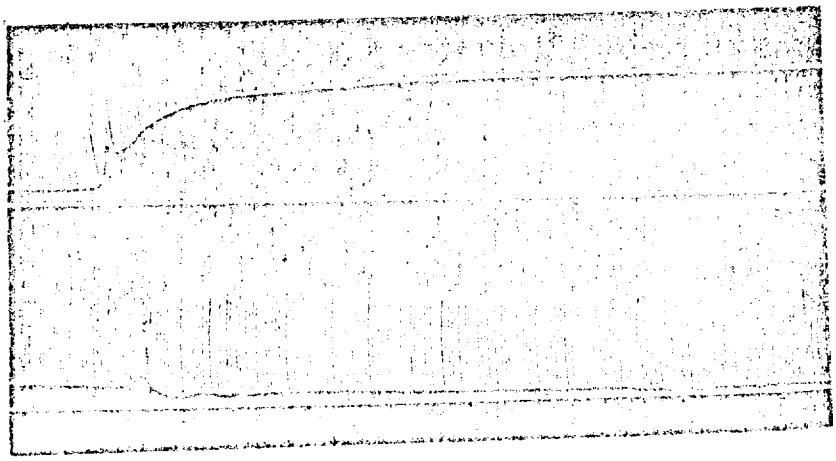

Fig. 8. Two biceps of the turtle. Indirect stimuli every 30 seconds, Above: 10 mg. of iodoacetamide. Below: 10 mg. of iodoacetamide plus 50 mg. of glucose.

Some of the substances that are mentioned as inhibitors of shortening produced by the sulfhydralic reagents are capable, by themselves, of producing rapid but reversible contractures, which permits the differentiation of their own effect from that of the reagent.

DISCUSSION

The comparative study of the properties of the different types of muscles and especially of the myocardium and the skeletal muscle includes a large number

of problems, one of which is attacked in the present article. Considering systolic arrest of the heart of cold-blooded animals as the shortening of a fiber produced by a toxic mechanism, we attempted to clarify the effect of the substances that produce it in the striated muscle, and is thus that we sought for the shortening of a skeletal muscle that would lend itself for this study. All the substances studied produced a shortening similar to systolic arrest which induces us to think that the inner mechanism of contraction in both types of muscle has great similarities. In greater abundance, we could observe an effect similar to positive inotropic effect that is seen in the heart although there was no electric registering of the contraction to let us know if this is due to multiple discharges as in the case of veratrinized muscles. A further confirmation is given by the fact that the action of the sulfhydralic reagents may be inhibited with glutathione and with cysteine just as in the heart. The inhibition on the effect of the sulfhydralic reagents of a large group of substances related to the metabolism of the glucides is interesting since what the reagents mentioned possibly produce is an interruption of this metabolism and, therefore a paralysation of the mobilization of phosphorus, which is so important for muscular contraction.

SUMMARY

The responses of some skeletal muscles were studied vis-à-vis the digitalic drugs and certain specific reagents of the sulfhydralic enzymes, comparing them with systolic arrest of the hearts in cold-blooded animals. A preparation of skeletal muscle is described that greatly facilitates the study of the action of substances on the said tissue.

The digitalic drugs and the sulfhydralic reagents produced an irreversible shortening in the muscles studied.

The shortening produced by the sulfhydralic reagents can be inhibited by glutathione, cysteine, glucose, sodium lactate, sodium acetate, ferric citrate, and glycerin.

The effect of the digitalic drugs is not inhibited by the substances mentioned in the previous sentence.

The mechanisms of muscular shortening caused by the action of the different drugs studied are discussed.

I thank Doctor Arturo Rosenblueth and Doctor Rafael Méndez for their aid and advice.

ACCION SOBRE EL MUSCULO ESTRIADO DE ALGUNAS SUBSTANCIAS QUE PRODUCEN PARO SISTOLICO DEL CORAZON

Arch. Inst. Cardiol. (Méx.)
18: 111-21 (1948)

J. Pisanty *

Introducción

En el presente trabajo se estudiaron los efectos sobre el músculo esquelético, de una serie de substancias cuya propiedad común es la de producir un efecto cardíaco caracterizado por una disminución progresiva de la relajación de la fibra, que termina con un paro en sístole de los corazones de los animales de sangre fría. Este estudio forma parte de un programa elaborado por el doctor Arturo Rosenblueth para comparar las propiedades de los diferentes tipos de tejido muscular y sus respuestas a las diversas substancias que actúan sobre los mismos.

La detención en sístole del corazón de la rana se consideró hasta hace pocos años como reactivo específico de los medicamentos digitálicos y sus glucósidos, del exceso de ión calcio y de las sales ionizables de bario. La especificidad de este fenómeno ha ido disvaneciéndose de día en día, desde que Méndez y Montes (1943) y Krayer, Méndez y Moe (1944), demostraron que diversos alcaloides obtenidos de algunas especies de Veratrum son capaces de producir parada sistólica del ventrículo de la rana y otros efectos que preceden a éste episodio terminal.

Según se ha observado recientemente por Méndez (1946), existe un grupo de substancias que no están relacionadas entre sí por su constitución química, pero que pueden agruparse por su caracteris-

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tica de combinarse con los grupos SH— de las enzimas sulfhidrílicas, y que son capaces también de producir un paro sistólico del corazón de ciertos animales de sangre fría. Este grupo de substancias, conocidas por el nombre general de reactivos sulfhidrílicos, habían sido ya estudiadas por Hellermann, Chinard y Deitz (1943) y por Guzmán Barrón y Singer (1945) en sistemas enzimáticos aislados. Méndez (1946) inició su estudio en tejidos vivos.

Tiene indudablemente un gran interés conocer el comportamiento del músculo esquelético frente a ese grupo de substancias cuya acción común es de producir paro sistólico en los corazones de los animales de sangre fría.

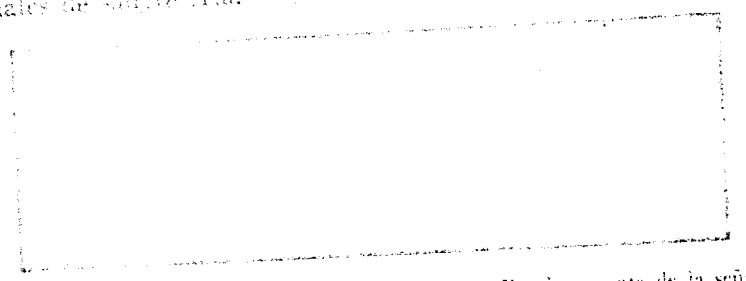

Fig. 1.—Bíceps de tortuga estimulado cada 30 segundos. En el momento de la señal se inyectó 0.5 mg. de ouabaína.

Método

En los resultados que exponemos a continuación se analizan los efectos sobre el músculo estriado de dos glucósidos cardíacos: digitoxina (digitalina Nativelle) y ouabaína, que pueden considerarse como representantes genuinos del amplio grupo de glucósidos de "acción digitálica", así como los de tres de los reactivos específicos de las enzimas sulfhidrílicas: ácido ortoyodosobenzoico, yodoacetamida y clorhidrato del ácido 3. amino. 4. hidroxibencenoarsenioso.

Se ensayó primero el sartorio de la rana, como lo había hecho McKeen Catell (1938) en un estudio de la acción de la estrofantina. Se ensayó asimismo el método utilizado por del Pozo (1943) en sus estudios sobre la acción de ciertas estrofantinas sobre el gastrocnemio circulado del gato. No fué posible con ninguno de los dos métodos citados obtener acortamiento muscular por efecto de las substancias empleadas, por lo que se buscó una preparación adecuada

en el sentido de que permita inyectar las substancias a un músculo cuyas contracciones se puedan registrar fácilmente y que además las inyecciones difundan lo menos posible al resto del animal, para lo cual se hizo necesario un músculo cuya circulación sea muy lenta, al mismo tiempo que tenga una arteria con suficiente luz para poder inyectar sin demasiadas dificultades.

A una tortuga descerebrada y sin la parte superior del caparazón se le disecan ambos bíceps, se ata la extremidad distal de éstos a un hilo suficientemente resistente y se fija éste al miógrafo isotónico inscriptor. Por otra parte, se clava un punzón en la articulación escapulohumeral y se fija a un soporte. Se corta la extremidad distal del bíceps, ya atada, dejándolo de este modo unido al animal solamente por su inserción en el hombro, sitio por donde penetran

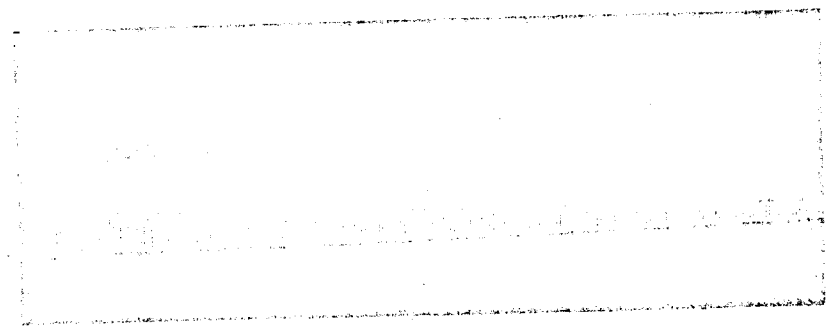

Fig. 2.—Dos bíceps de tortuga. Al del trazo superior se le han inyectado, en dosis fraccionadas, 0.6 mg. de digitoxina. El trazo inferior es el control sin inyección. Estímulos indirectos de 0.05 microfaradios a 30 volts, cada 30 segundos. Tiempo: 1 min.

sus vasos nutricios. Se diseca la arteria subclavia. Se busca por debajo del omoplato el plexo braquial, se le diseca, se machaca su extremo proximal para evitar la llegada de impulsos nerviosos a los músculos que inerva y se le colocan electrodos, que servirán para estimular. Se corta la extremidad desde cerca de la articulación del hombro para evitar el registro de sus movimientos, que pueden interferir con los del bíceps.

Con esta preparación es posible inyectar a los músculos escapulares irrigados y al bíceps, sin producir la muerte del animal, cantidades de las substancias mencionadas muy superiores a las que es posible inyectar a mamíferos sin obtener un efecto adecuado.

Las soluciones de todas las substancias que se inyectaron fueron previamente ajustadas a un pH de 7.5. Siempre que fué posible se inyectaron a una concentración osmótica aproximada a la del líquido de Ringer.

La concentración usada para los inhibidores de los reactivos de las enzimas sulfhidrílicas fué siempre 5 a 10 veces mayor, en peso, que la del reactivo ensayado; e. g., para 10 mg. de yodoacetamida, 100 mg. de acetato de sodio.

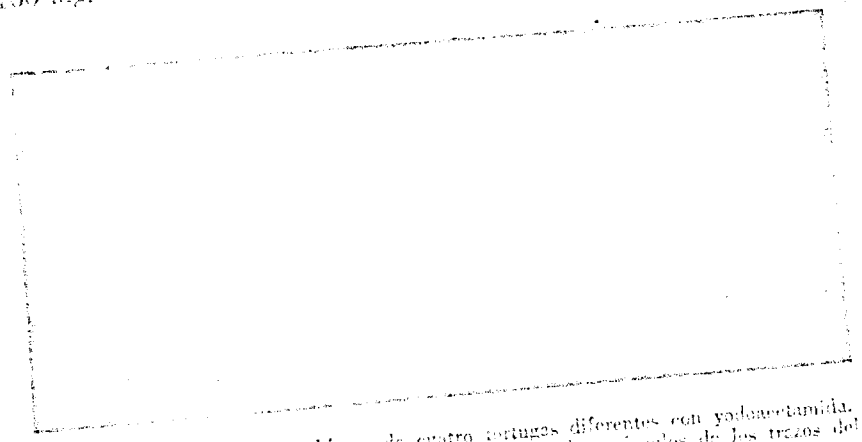

Fig. 3.—Acortamiento de los bíceps de cuatro tortugas diferentes con yodoacetamida. Las dosis usadas fueron de 100, 25, 10 y 2 mg., para los músculos de los trozos del superior por, [illegible] y sucesivamente. Previamente se ha logrado que las respuestas a un estímulo dado sean iguales, modificando el brazo de palanca.

Resultados

A) *Glucósidos cardíacos.* a) *Ouabaína.* Al inyectar dosis desde 0.05 mg., se obtiene un acortamiento del músculo, acortamiento que es irreversible, progresivo y que alcanza una altura superior a la contracción máxima que da un choque único indirecto. Dosis menores no produjeron acortamiento ni tuvieron efecto después de la aplicación de estímulos al nervio. Cuando se hicieron estimulaciones periódicas se observó un efecto semejante al inotrópico positivo que se registra en los corazones de los animales de sangre fría. El efecto de 0.5 mg. de ouabaína en un bíceps de tortuga se ilustra en la figura 1.

b) *Digitoxina.* En esencia, la acción de esta substancia es igual a la de la ouabaína. Por la dificultades que tiene el disolverla, se inyectó habitualmente en dosis fraccionadas. En la figura 2 se ve la acción de 0.6 mg. de digitoxina inyectados a dosis fraccionadas, comparando con un músculo al cual no se ha inyectado y se ve un aumento de la amplitud de las contracciones.

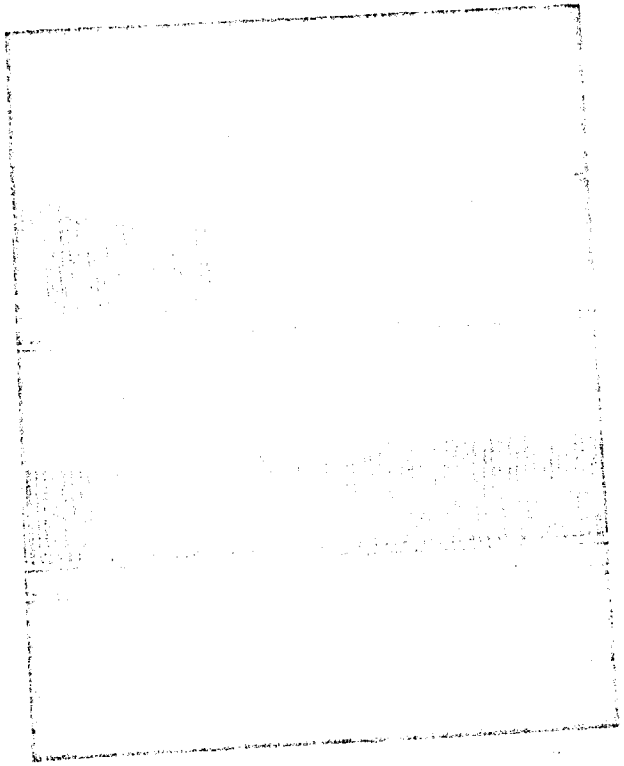

Fig. 1.—Ambos gastrocnemios de gato. Estímulos indirectos cada 30 seg. Al del registro superior se le inyectó 200 mg. de yodoacetamida. Nótese el efecto inotrópico positivo. El registro inferior es del músculo control, que no se inyectó.

Es interesante que el acortamiento producido con estas drogas fué siempre máximo y que no se pudieron obtener acortamientos intermedios variando la dosis. No fueron necesarios estímulos para desencadenar este fenómeno.

c) Otras substancias. Tanto la ouabaína como la digitoxina se inyectaron junto con glutatión, cisteína, glucosa, lactato de sodio y acetato de sodio, sin obtener modificaciones de sus efectos. Tampoco se pudo impedir su acción con atropina, lo cual sí se logra en ciertas condiciones en el músculo liso (Alanís, 1947).

B) *Reactivos sulfhidrílicos.* a) *Yodoacetamida.* Esta substancia produce con facilidad acortamientos irreversibles del músculo esquelético, precedidos de un claro aumento de la amplitud de las contracciones. Para el bíceps de tortuga, dosis de 1 a 2 mg. son inefectivas, y ya dosis de 4 a 5 mg. producen acortamiento, siendo

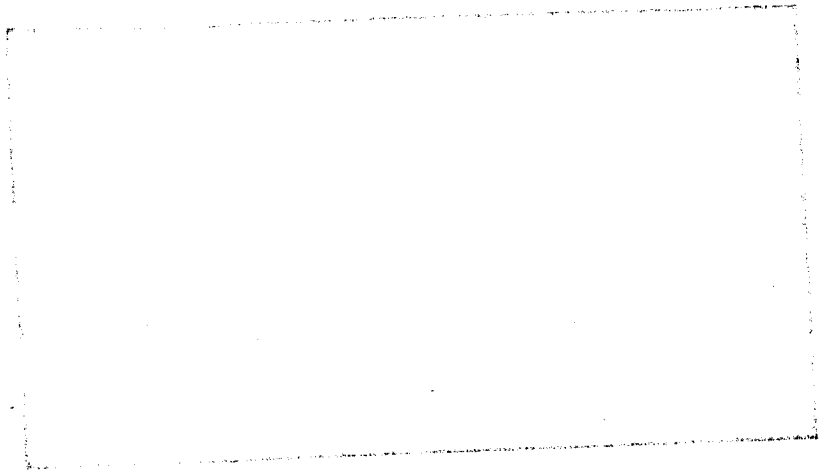

Fig. 5.—Influencia de la frecuencia de estimulación. Son dos bíceps de tortuga a los que se inyectó la misma cantidad de yodoacetamida. Al superior se le aplican estímulos indirectos cada cinco minutos y al inferior no se le estimula.

éste mayor, mientras mayor sea la cantidad inyectada, como puede apreciarse en la figura 3, con dosis de 2, 10, 20 y 100 mg. Este reactivo es el único con el cual se pudo reproducir el acortamiento en el gastrocnemio del gato con dosis de 200 mg., con aumento de la amplitud muy claro, como se aprecia en la figura 4. Varios ensayos, en gato, hechos con dosis menores, no dieron resultado.

Tanto la yodoacetamida como los reactivos estudiados en *b*) y en *c*) producen un acortamiento espontáneo, es decir, que no necesita de estímulos para desencadenarse; además no es influído por la

frecuencia de la estimulación. Así, en la figura 5 se ve el efecto semejante de cantidades iguales de yodoacetamida, con y sin estimulación.

b) *Ácido o-yodosobenzoico.* Las dosis mínimas necesarias de este reactivo son ligeramente superiores a las de yodoacetamida y, a semejanza de ésta, produce un acortamiento más intenso a medida que la dosis es mayor, como se ilustra en la figura 6. No son necesarios estímulos para desencadenar su acción y ésta no es reversible.

c) *Clorhidrato del ácido 3. amino. 4. hidroxifenilarsenioso.* * Las dosis mínimas de este reactivo para obtener disminución en la longitud del bíceps de la tortuga fueron de 120 a 150 mg. La figura

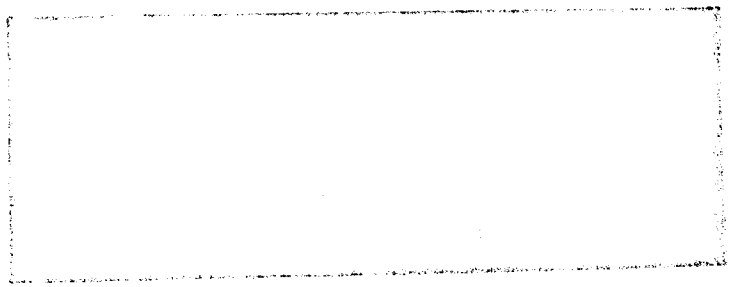

Fig. 6.—Bíceps de tortuga. Las respuestas a un estímulo han sido previamente igualadas variando la longitud del brazo de palanca. El trazo superior es de un bíceps inyectado con 20 mg. de ácido o-yodosobenzoico, el medio con 10 mg. y el inferior con uno.

7 es el registro del acortamiento producido por una dosis de 200 mg.

Esta substancia es la de más difícil estudio de todas las que se usaron, por la gran concentración a que es necesario emplearla y por las dificultades inherentes a su neutralización.

d) *Agentes inhibidores.* El acortamiento producido por los reactivos sulfhidrílicos no revierte espontáneamente, ni ha sido posible hacerlo reversible con ninguno de los inhibidores de reactivos sulfhidrílicos estudiados. Este acortamiento es, sin embargo, prevenido por la adición de grupos SH— en forma de cisteína o de glutatión y por diferentes substancias relacionadas con el metabolismo intermediario de la glucosa y por la glucosa misma, como se ve en la figura 8.

* Este reactivo, en forma anhidra, fué generosamente suministrado por la casa Parke Davis y Co.

De estas substancias relacionadas con el metabolismo intermediario de la glucosa, se ensayaron el lactato de sodio, el acetato de sodio, citrato férrico y glicerina, todos los cuales son capaces de impedir la acción de los reactivos sulfhidrílicos.

Fig. 7.—Arriba: músculo control, no inyectado. Su trazo es paralelo a la línea de los ceros. Abajo: músculo al cual se le han inyectado 200 mg. de clorhidrato del ácido 3-amino, 4-hidroxibencenoarsenioso en solución neutra.

Fig. 8.—Dos bíceps de tortuga. Estímulos indirectos cada 30 seg. Arriba: 10 mg. de yodoacetamida. Abajo, 10 mg. de yodoacetamida más 50 mg. de glucosa.

Algunas de las substancias que se mencionan como oponentes al acortamiento producido por los reactivos sulfhidrílicos son capaces, por sí solas, de producir contracturas rápidas pero reversibles, lo cual permite diferenciar su acción propia con la del reactivo.

Discusión

El estudio comparativo entre las propiedades de los diferentes tipos de músculos y en especial entre miocardio y músculo esquelético, encierra un gran número de problemas, uno de los cuales se ataca en el presente trabajo. Considerando el paro en sístole del corazón de los animales de sangre fría como el acortamiento de una fibra producido por un mecanismo tóxico, se trató de aclarar la acción de las substancias que lo producen, en el músculo estriado, y es así como se ha buscado el acortamiento de un músculo esquelético que se preste para este estudio. Todas las substancias estudiadas produjeron un acortamiento semejante al paro sistólico, lo que nos induce a pensar que el mecanismo íntimo de la contracción en ambos tipos de músculo tiene grandes semejanzas. A mayor abundamiento, se pudo observar un efecto semejante al efecto inotrópico positivo que se ve en el corazón, aunque no se hizo registro eléctrico de la contracción para saber si esto es debido a descargas múltiples, como cuando se trata de músculos veratrinizados. Una confirmación más la proporciona el hecho de que se impida la acción de los reactivos sulfhidrílicos con glutatión y con cisteína, igual que en el corazón.

Es interesante la oposición a la acción de los reactivos sulfhidrílicos, de un grupo grande de substancias relacionadas con el metabolismo de los glúcidos, ya que lo que posiblemente producen los reactivos mencionados es una interrupción de este metabolismo y, por lo tanto, una paralización de la movilización del fósforo, tan importante para la contracción muscular.

Resumen

Se estudiaron las respuestas de algunos músculos esqueléticos frente a las drogas digitálicas y a ciertos reactivos específicos de las enzimas sulfhidrílicas, comparándolas con el paro sistólico de los corazones de los animales de sangre fría.

Se describe una preparación de músculo esquelético que facilita grandemente el estudio de la acción de substancias sobre dicho tejido.

Las drogas digitálicas y los reactivos sulfhidrílicos producen un acortamiento irreversible en los músculos estudiados.

El acortamiento producido por los reactivos sulfhidrílicos se puede prevenir por glutatión, cisteína, glucosa, lactato de sodio, acetato de sodio, citrato férrico y glicerina.

El efecto de los digitálicos no se previene por las substancias mencionadas en el inciso anterior.

Se discuten los mecanismos del acortamiento muscular bajo la acción de las diferentes drogas estudiadas.

Agradecemos al Dr. [illegible] su ayuda y consejos.

Résumé

Nous avons étudié les réactions de quelques muscles striés aux drogues à base de digitale et de certains produits spécifiques du groupe des enzymes sulphohydryliques, les comparant avec l'arrêt systolique des cœurs des animaux à sang froid.

Nous donnons la description d'une méthode pour préparer le muscle et faciliter considérablement l'étude de l'action de certaines substances sur le tissu musculaire.

Les drogues à base de digitale et les composés sulphohydryliques provoquent un raccourcissement irréversible des muscles striés.

Le raccourcissement provoqué par ces composés peut être prévenu par le glutathion, la cysteine, la glucose, le lactate et l'acétate de sodium, le citrate de fer et la glycérine.

L'effet de la digitale ne peut pas être prévenu par les substances mentionnées dans le chapitre précédent.

Nous discutons le mécanisme du raccourcissement des muscles sous l'effet des diverses drogues étudiées.

Summary

The response of some striated muscles to drugs of the digitalis group and to some specific reactives of the sulphydril enzimes has been studied. A comparison has been made between this response and the systolic standstill of the hearts of cold blooded animals.

A new preparation of striated muscle is described: this preparation facilitates greatly the study of the action of different substances on the tissue.

Digitalis drugs and sulphydril reactives produce an unreversible shortening of the muscles employed in this work.

The shortening produced by the sulphydril reactives may be prevented by glutation, cistein, glucose, sodium lactate, sodium acetate, ferric citrate and glycerin.

The effect of digitalis drugs is not prevented by the substances mentioned above.

The mechanisms of the muscular shortening under the action of the different drugs used in this study are discussed.

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THE INFLUENCE OF SOME IRRITANT CHEMICALS AND SCARIFICATION ON TUMOUR INITIATION BY URETHANE IN MICE

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When mice were injected with urethane and the skin of the back subsequently painted once each seventh day for twenty weeks with croton oil as a promoting agent, the number of papillomata produced was found to be increased if the mice were given a preliminary application of croton oil, dissolved in acetone, to the area at 18, 24 or 48 hours preceding the injection of urethane (Pound and Bell, 1962). This was due to local phenomena produced in the skin by the preliminary application of croton oil (Pound, 1963).

Croton oil is one of the few materials that act as potent promoting agents after the application of initiating agents such as the carcinogenic hydrocarbons is small single doses to the skin of mice (Gwynn and Salaman, 1953; Roe and Peirce, 1961). Local applications of many other substances that, like croton oil, produce inflammation or hyperplasia of the skin did not promote the development of tumours in this animal (Salaman, 1958).

It was necessary, therefore, to consider whether the augmenting influence of a preliminary application of croton oil was associated with croton oil specifically, in particular with its action as a promoter, or was an effect that could be elicited by any other substance or means that produced inflammation or cellular proliferation in the skin. Some authors consider that croton oil itself is a mild carcinogen (Roe, 1956; Boutwell, Bosch and Rusch, 1957) and, following this line of thought, it is possible that the augmenting influence of croton oil, applied before the administration of urethane, might be viewed as a simple additive effect or due to synergism. The time relationships reported by Pound and Bell (1962) disprove the first of these views and render the second improbable except in a general sense lacking scientific precision.

Accordingly, the present experiments were carried out to determine the influence of scarification or preliminary treatment of the skin with various chemicals that produce hyperplasia or inflammation (referred to collectively in this paper as "irritants") on the initiation of tumours by urethane. Further, the augmenting influence of a solution of croton oil in acetone has been ascribed in previous work to the croton oil (Pound and Bell, 1962; Pound, 1963) so that data are now presented formally proving that the acetone solvent has an insignificant influence in this respect.

MATERIALS AND METHODS

Mice

Mice of the strain "Hall" bred in this department (Pound, 1962) were used. The animals weighed between 20 and 30 grams at the beginning of the experiments and were accommodated in stainless steel compartments each holding ten mice.

Bedding was provided as a layer of coarse sawdust that was changed weekly. The mouse room was air conditioned at 22° C.

The animals were fed a diet containing 18 per cent protein, 55 per cent carbohydrate, 11 per cent fat, 11 per cent fibre with an added vitamin and mineral supplement. The diet and water were made available in excess of the animals' needs. The hair of the skin of the back was clipped before each application of croton oil or other treatment.

Chemicals

For standard initiation-promotion treatment.—Urethane, British Drug Houses, Laboratory Reagent grade; Acetone, Univar, Analytical Reagent grade; Croton oil, Stafford, Allen & Sons, London.

Irritants selected.—Acetic acid, Osta Chemicals, Analytical Reagent grade. Turpentine, vegetable turpentine, Drug Houses of Australia. Xylene, Unilab., B.P. 137–142° C., neutral, non-volatile matter less than 0·02 per cent, sulphur less than 0·0003 per cent. Trichloroacetic acid, Univar, Analytical Reagent grade. Iodoacetic acid, British Drug Houses, Laboratory Reagent grade. Cantharidin, crystalline, Taylors Elliotts and Australian Drug Houses Ltd.

Urethane was injected as a solution in isotonic saline containing 25 mg. per 0·5 ml., sterilised by Seitz filtration. The injections were made subcutaneously between the scapulae. The irritants were used either undiluted or as solutions in acetone as indicated in Table I.

TABLE I.—*Schedule of Experimental Treatments*

Experiment	Groups	Preliminary treatment before injection of urethane
I	1–8	Acetone, undiluted, 0·25 ml.
23 June 1962*	9–12	Acetone, undiluted, 0·25 ml.
II	13–18	Acetic acid, 50 per cent (v/v) solution in acetone, 0·25 ml.
	19–24	Xylene, undiluted, 0·25 ml.
27 August 1962*	25–30	Vegetable turpentine, undiluted, 0·25 ml.
III	31–34	Cantharidin, 0·1 per cent (w/v) solution in acetone, 0·25 ml.
7 November 1962*	35–38	Iodoacetic acid, 1·0 per cent (w/v) solution in acetone, 0·25 ml.
	39–42	Trichloroacetic acid, 5·0 per cent (w/v) solution in acetone, 0·25 ml.
	43–46	Mechanical abrasion, as described in text.

* = Dates of injection with 25 mg. urethane.

EXPERIMENTAL

Male mice were grouped at random into groups of twenty, with the provision for some mortality noted below in the case of the groups treated with cantharidin. The stage of the hair cycle was ignored.

Experiment I

The mice of groups 1 to 8 (Table I) were given no preliminary application or a preliminary application of acetone to the whole area of the skin of the back at 0, 12, 18, 24 hours, 2, 4 or 6 days before an injection of 25 mg. urethane, respectively. The animals of groups 9 to 12 were given an application of 0·25 ml. acetone

to the right side of the skin of the back at 24 hours prior to the injection of 25 mg. urethane.

Experiments II and III

In random lots, the mice of six groups in Experiment II or of four groups in Experiment III, were given a preliminary treatment with one of the various irritants or scarification to the right side of the skin of the back at intervals of 1, 2, 3, 4, 5 or 6 days in Experiment II, or 1, 2, 3 or 5 days in Experiment III, respective to the groups, before the injection of 25 mg. urethane according to the schedule in Table I. Thus, for example, groups 13, 14, 15, 16, 17 and 18 were given a single application of 0·25 ml. of a 50 per cent solution of acetic acid in acetone to the right side of the skin of the back at intervals of 1, 2, 3, 4, 5 or 6 days respectively before the injection of urethane: and so on *mutatis mutandis* for each lot of six or four groups with each preliminary treatment.

In each experiment, from the seventh day after the injection of urethane the mice were painted over the whole area, that is both right and left sides, of the skin of the back with approximately 0·25 ml. of a 0·5 per cent solution of croton oil in acetone once each seventh day for twenty weeks.

At the time of each painting, or after the sixteenth week fortnightly, the numbers of survivors, the number of papillomata and their distribution on the skin of the back were recorded. The final count was made two weeks after the last application of croton oil.

The concentrations of the various irritants used for the preliminary treatment were based on the work of Berenblum (1935, 1941) concerning the influence of various chemicals when applied with repeated applications of carcinogenic hydrocarbons. Preliminary experiments were made to determine the effects of various concentrations on mouse skin and concentrations selected that resulted in considerable hyperplasia and scaling of the skin without gross ulceration. However, the mice treated with 50 per cent acetic acid were observed only for 6 days by which time ulceration had not occurred but, in the experiment proper, gross ulceration invariably occurred later. In the case of iodoacetic acid and cantharidin, higher concentrations also led to considerable mortality in the mice. Even with the concentration of cantharidin used, allowance had to be made for a 1 in 6 mortality to provide a full complement of twenty mice after one week, extra mice being disposed of at random before the first weekly application of croton oil.

Mechanical abrasion was carried out with the aid of the saw edge of a hacksaw blade held vertically to the skin with the long axis at right angles to the line of abrasion. With the clipped skin of the mice drawn taut, the blade was moved cranially and caudally along the area to be abraded until the superacial layers of skin were removed and the surface was moist and shiny due to serous oozing. The abrasion was usually not deep enough to produce bleeding. Alternative methods tested for abrasion using fine, medium or coarse glass paper were not as satisfactory because of the mobility of the skin.

RESULTS

Clinical effects of the irritants and scarification

All the preliminary treatments produced acute inflammation in the treated area of skin. Oedema was apparent within 6 hours and developed most rapidly

in the case of scarification, iodoacetic acid and cantharidin and least rapidly with turpentine. Twenty-four hours after the application the epidermis was less translucent probably because of epidermal proliferation since this appearance gradually gave place by the third or fourth day to a scaling hyperkeratosis that varied considerably in intensity between the different treatments. The oedema and epidermal changes were in all cases confined to the right side although often reaching the midline. No changes were observed on the left side of the skin of the back that had had no preliminary treatment.

The mice treated with cantharidin, iodoacetic acid or xylene in the amounts given showed some evidence of general toxic effects due to the drugs.

Acetic acid.—The treated skin was oedematous for about four days after application. By the third day the painted side appeared to be very tender to even light touch. A dry epithelial crust formed about the fourth day which separated from the sixth day onwards leaving an ulcerated surface that healed by the end of the third week. The extent of ulceration varied considerably but it was present in all mice at some time within the first two weeks. Healing of the ulcerated areas resulted in linear scars easily seen for the duration of the experiment as ridges of tissue in which short hairs persisted after clipping had removed the surrounding hair.

Xylene.—The oedema was easily observed in all the mice for four days. From the third day, dry scaliness became apparent and was present for about one week. The mice of these groups became very irritable after the application of xylene and engaged in a considerable amount of back-biting. No ulceration appeared to result from the xylene itself. The xylene appeared to have some toxicity for the mice, eight of which died in the two weeks after the application for no other cause.

Vegetable turpentine.—This produced the mildest response of all the irritants used. Oedema persisted for two to three days and scaling of the treated skin occurred from about the third to seventh day, but this was less than that produced by any of the other treatments.

Cantharidin.—All surviving mice showed severe oedema and tenderness for about five days in the painted area and the skin felt "leathery" between the palpating fingers. All the mice developed gross dry scaly hyperkeratosis from the third day. A few mice developed small areas of localized ulceration but there was no subsequent scarring. During the second week most mice lost hair in the area painted. The degree of epilation varied from complete baldness to only small hairless islands. Many remained hairless for four weeks and a few for longer. The mice of these groups also showed a generalized malaise, their eyes being a little sunken and their tails cold.

Iodoacetic acid.—Severe oedema and a curious pallor of the treated skin was apparent within 24 hours and was evident until the fourth day. Thereafter the affected skin remained thickened, firm and scaly for several days. At no stage did ulceration occur and by the end of two weeks the skin was similar to that on the untreated side. Three mice died from toxic effects of the drug within 24 hours of the application.

Trichloroacetic acid.—This produced considerable thickening and scaliness of the treated area of skin. There was no ulceration in the first seven days but during the second week a few small ulcers appeared on a few of the mice. This was not followed by scarring.

Mechanical abrasion.—Ulceration and oedema were subsiding and hair growth

was apparent within two days of abrasion and the whole surface was re-epithelialized in most cases after six days. By two weeks the skin was healed but with some areas of flat pale scarring. This scarring was visible for the duration of the experiment but was not present in prominent ridges as was the scarring after acetic acid. During the first few days these mice consumed twice the normal amount of water.

From these macroscopic observations a subjective assessment of the relative severity of the local effects of the preliminary treatments was made before counting the tumour yields. In decreasing order acetic acid, mechanical abrasion, cantharidin and iodoacetic acid produced the most severe changes, trichloroacetic acid and xylene appeared to produce a similar less effect and turpentine produced the least severe changes. However the character of the sequence of changes varied with the different irritants, thus iodoacetic acid, cantharidin and xylene produced more oedema than trichloroacetic acid or acetic acid although the latter produced more ulceration especially in the later stages, so that a precise comparison of the effects was not possible. It is emphasised that this grading refers only to the effects actually produced by the various preliminary treatments in the concentrations employed. Acetone produced no obvious change in the skin at all.

It became obvious from as early as the tenth week of painting with croton oil that when tumours developed there was a preference for the side that had received the preliminary treatments in Experiments II and III, whereas in Experiment I no obvious influence of the prior application of acetone alone was apparent at any stage.

Yield of Tumours

The number of mice surviving after 22 weeks and the yield of tumours in the various groups are shown in Tables II and III.

Although considerable care was taken to ensure that the preliminary treatments were confined to the right side, the possibility that some spread might have

TABLE II.—*Influence of a Preliminary Application of Acetone before Injection of Urethane*

Group	Interval between preliminary application and injection of urethane	Survivors	Mice with tumours: Number of mice	Number of tumours: Right side	Midline	Left side	Total
1	No prior application	15	8	4	0	6	10
2	0	13	7	4	1	6	11
3	12 hours	12	5	3	0	4	7
4	18 ,,	15	6	5	0	4	9
5	24 ,,	18	6	4	2	6	12
6	2 days	17	4	5	2	3	10
7	4 ,,	14	2	2	0	3	5
8	6 ,,	19	6	5	1	4	10
	Total	123	44	32	6	36	74
9, 10, 11, 12	24 hours	67	19	22	5	14	41

20 mice in each group at beginning of experiment.

extension to the opposite side cannot be rigidly excluded. In fact, as noted above, changes in the skin following the preliminary treatments did appear to be confined to the right side. Even if this were so, any influence of the preliminary treatments would be manifest in the mid-line zones between the treated and untreated sides and also, in a proportion of the mice, some tumours would have appeared in this zone without prior stimulation so that the assignation of mid-line tumours is not possible. Since the purpose of the experiment was to compare an area of skin that had a preliminary treatment with an area that had no such treatment, it is reasonable and best to exclude the mid-line tumours from consideration and compare the two sides that represent similar areas differing only in the preliminary treatment.

Therefore, in Tables II and III, the data have been dissected, from the recorded distribution of the lesions, to show the number of tumours on the right side, on the left side and on the mid-line of the skin of the back. For this purpose the mid-line was considered as a band about 5 mm. wide separating the two sides.

It is clear from Table II that there is no significant variation in the number of tumours between the groups of mice given a preliminary application of acetone at various intervals before the injection of urethane ($\chi^2 = 3{\cdot}09$, 7 d.f., N.S.) nor between the number of tumours on each side of the skin of mice given a preliminary application of acetone to the right side 24 hours before injection of urethane ($\chi^2 = 1{\cdot}7$, 1 d.f., $0{\cdot}20 > P > 0{\cdot}10$), nor between the two sides of the mice in general.

In the case of the mice treated on the right side with the various irritants or scarification, Table III shows clearly that the number of tumours on the treated side is significantly greater than on the untreated side with each of the seven treatments. It is also evident that the number of mice with tumours on the treated side is greater than the number of mice with tumours on the untreated side, and that the number of tumours per mouse likewise is increased on the treated side. However, since these parameters are at least partly related to the number of tumours they are not considered in the further analysis.

The analysis is based on the number of tumours recorded on each side for each individual mouse. As the distribution of the number of tumours per mouse is probably Poisson, the square root transformation was used throughout. The score of each mouse was then the square root of the number of tumours on the left side or the right side as the case may be.

Analysis of left sided tumours

To test whether the unpainted sides had been affected by the treatments an analysis of the left side scores was made for the seven treatments in Experiments II and III and for days −1, −2, −3 and −5 giving a 7 by 4 factorial arrangement with unequal class numbers. There was a significant difference between the effects of the different treatments ($P < 0{\cdot}005$). The days effect was not significant nor was there any significant interaction between treatment and days.

The groups of mice treated with cantharidin, iodoacetic acid and xylene gave significantly higher yields than the groups treated with acetic acid, trichloroacetic acid, turpentine or scarification. Within the first group cantharidin gave a significantly higher yield than iodoacetic acid or xylene. These tests were performed at the 5 per cent level of significance.

TABLE III.—*Influence of Preliminary Treatment with Irritant Chemicals or Scarification before Injection of Urethane*

Preliminary treatment	Group	Interval between preliminary treatment and injection of urethane	Number of survivors	Mice with mid-line tumours: Number of mice	Mice with mid-line tumours: Number of tumours	Mice with no tumours	Distribution of tumours per mouse on treated and untreated sides (Number of tumours on right side / Number of tumours on left side)
Acetic acid	13	−1	17	3	4	7	4/1 2/1 1/1 4/0 3/0 2/0 1/0 1/0 1/0 0/1
	14	2	15	0	0	5	3/1 2/1 4/0 4/0 3/0 2/0 1/0 1/0 1/0 0/1
	15	−3	13	2	2	2	6/2 3/2 11/1 1/1 4/0 3/0 3/0 2/0 1/0 1/0
	16	−4	17	0	0	7	1/2 6/0 4/0 3/0 2/0 2/0 1/0 1/0 1/0 0/1
	17	−5	17	0	0	7	2/1 1/1 4/0 2/0 2/0 1/0 1/0 1/0 1/0 0/1
	18	−6	17	1	2	10	1/2 1/0 1/0 1/0 1/0 0/1 0/1
Xylene	19	−1	18	4	5	2	4/3 7/2 5/1 3/1 3/1 3/0 3/0 3/1 2/0 2/0 2/0 2/0 1/0 1/0 1/0 1/0
	20	−2	12	4	4	1	6/2 1/3 4/2 4/1 3/1 10/0 4/0 4/0 1/0 1/0 1/0
	21	−3	17	4	4	7	11/1 3/0 3/0 2/0 2/0 2/0 1/0 1/0 0/1 0/1
	22	−4	17	4	6	8	1/3 3/1 2/1 3/0 3/0 3/0 2/0 1/0 1/0
	23	−5	14	0	0	5	8/3 3/2 3/1 5/0 3/0 2/0 1/0 0/1 0/1
	24	−6	15	1	1	6	2/0 2/0 2/0 1/0 1/0 1/0 1/0 1/0 1/0
Turpentine	25	−1	17	1	1	10	5/1 5/1 3/1 2/1 1/1 4/0 1/0
	26	−2	18	1	1	5	4/2 3/1 1/1 4/0 3/0 3/0 2/0 2/0 1/0 1/0 1/0 1/0 1/0
	27	−3	19	1	1	9	5/0 4/0 4/0 3/0 1/0 1/0 1/0 0/1 0/1 0/1
	28	−4	19	2	2	10	3/2 1/1 2/0 2/0 1/0 1/0 1/0 0/2 0/1
	29	−5	19	1	1	12	3/0 2/0 1/0 1/0 1/0 0/1 0/1
	30	−6	18	0	0	12	1/1 2/0 1/0 1/0 0/1 0/1
Cantharidin	31	−1	16	5	5	3	4/1 2/2 9/1 6/1 3/1 2/1 1/1 2/0 2/0 1/0 1/0 0/1
	32	−2	20	3	3	3	10/2 1/2 4/3 11/1 2/1 1/1 8/0 8/0 7/0 6/0 3/0 3/0 2/0 1/0
	33	−3	19	9	11	2	9/3 3/3 7/2 4/2 3/2 2/2 2/2 6/1 6/1 5/1 5/0 4/0 4/0 2/0 1/0 0/1
	34	−5	20	3	5	7	10/2 4/2 2/1 2/1 3/0 3/0 2/0 2/0 2/0 1/0 1/0 0/1
Iodoacetic acid	35	−1	19	3	3	7	11/2 4/2 11/1 5/1 3/1 6/0 5/0 2/0 1/0 1/0 1/0 1/0
	36	−2	19	3	3	5	8/2 7/2 2/2 1/2 9/1 7/1 4/1 1/1 2/0 2/0 2/0 1/0 0/1 0/1
	37	−3	17	2	3	4	9/2 5/2 4/2 6/1 3/1 2/1 8/0 6/0 3/0 2/0 2/0 1/0 1/0
	38	−5	18	1	2	6	3/3 1/2 5/1 3/1 3/1 3/1 1/1 2/0 1/0 1/0 1/0 1/0
Trichloroacetic Acid	39	−1	18	1	2	11	2/0 2/0 2/0 1/0 1/0 1/0 1/0
	40	−2	19	2	2	5	6/2 6/2 4/1 2/1 2/1 1/1 2/0 2/0 2/0 2/0 2/0 1/0 1/0 1/0 0/2 0/1
	41	−3	18	4	4	5	4/1 1/1 6/0 4/0 3/0 3/0 3/0 1/0 1/0 1/0
	42	−5	16	1	1	6	3/1 2/0 1/0 1/0 1/0 1/0 0/1 0/1 0/1 0/1
Scarification	43	−1	16	2	2	3	4/1 2/1 1/1 3/0 3/0 3/0 3/0 3/0 2/0 2/0 1/0 1/0 0/1
	44	−2	17	2	2	3	3/2 2/2 6/1 3/1 2/1 1/1 7/0 4/0 3/0 3/0 3/0 2/0 2/0 2/0
	45	−3	15	1	1	5	4/1 1/1 4/0 3/0 2/0 1/0 1/0 1/0 1/0 1/0
	46	−5	17	2	2	10	5/1 2/1 3/0 1/0 0/1 0/1 0/1

Analysis of right-sided tumours

The yield of tumours on the right side showed a significant correlation with the yield on the left side. To examine the way in which the treatments produced their effects an analysis of covariance was undertaken for the 3 by 6 factorial involving acetic acid, xylene and turpentine, Experiment II, and a similar analysis for the 4 by 4 factorial involving cantharidin, iodoacetic acid, trichloroacetic acid and scarification, Experiment III.

In the first analysis it was found that after the correlation with the left sides was eliminated, the treatment effect was still significant ($P < 0{\cdot}005$). There were significant differences between days ($P < 0{\cdot}005$) but there was no interaction between days and treatments. The treatment means were adjusted for regression on the left-hand side yield and these adjusted means were ranked in descending order as xylene, acetic acid and turpentine. The differences between each pair were found to be significant. The maximum adjusted mean for the day's effect occurred for day −2.

For the second set of treatments, Experiment III, the covariance analysis again showed a significant treatment effect ($P < 0{\cdot}05$), a significant day's effect ($P < 0{\cdot}005$) and no significant interaction between days and treatments. The adjusted treatment means were ranked in descending order as cantharidin, iodoacetic acid, scarification and trichloroacetic acid. The appropriate tests then showed that the adjusted yields for cantharidin and iodoacetic acid were significantly greater than for trichloroacetic acid, no other treatment differences being significant. The maximum day effect was for day −3 followed closely by day −2.

Care must be taken in interpreting analyses of covariance when the control variable (that is the tumour yield on the side that had no preliminary treatment) has apparently been effected by the treatments. If it is assumed that there was a systemic effect which was reflected in the variation of the left side tumours from treatment to treatment, this effect is presumably removed by the covariance analysis. The significant variability that remains among the adjusted treatment means indicates that the postulated systemic effect does not measure the whole of the relative effects of the different treatments but that the direct effects on the painted sides still vary from treatment to treatment in the manner described.

Time of appearance of tumours

The mean of the times of appearance of each tumour on the untreated side was 16·4 weeks over all the mice in all the groups and varied from 13 weeks to 20 weeks for the mice of the individual groups, whereas on the treated side it was 14·5 weeks over all the groups and varied from 12 weeks to 19 weeks between the groups. However the mean times of appearance of the tumours would appear to to partly related to the other parameters and since it is doubtful if differences of this order would be of biological significance in experiments of this design, no statistical analysis has been undertaken. As would be expected, the first tumours to appear occurred earlier on the treated side but this was not invariably the case.

Distributions of papillomata

In general the papillomata appeared to be randomly distributed over the skin of the different areas considered. However, in the case of groups 13 to 18

in which the mice were treated with acetic acid on the right side, there was an obvious concentration of papillomata along the margins of the prominent linear scars, 37 of the 116 tumours on the treated side appearing in this situation. There was no demonstrable relationship between the site of development of tumours and the pale flat scars on the right side of the skin of the back of the mice subjected to mechanical abrasion (groups 43 to 46).

DISCUSSION

In the first place, Experiment I shows that a single application of acetone to the skin of mice at various intervals preceding the injection of urethane has no significant influence on the tumour yield after subsequent promoting treatment with croton oil. The augmenting influence of a solution of croton oil in acetone (Pound, 1962; Pound and Bell, 1962; Pound, 1963) is therefore to be ascribed firmly to the croton oil. In this respect it is significant that acetone produced no clinical evidence of inflammation or cellular proliferation in the skin and that Pound (1962) found no significant histological change in the skin of mice that had been given twenty weekly applications of acetone. As a corollary, in view of the results with various irritants, it follows that even a small amount of a highly irritant impurity in the acetone might, in certain circumstances, lead to an augmented tumour yield.

Secondly, in contrast to the lack of any influence of the acetone solvent, Experiments II and III show that the yield of tumours was increased by preliminary treatment of the skin with acetic acid, xylene or turpentine in Experiment II, or with cantharidin, iodoacetic acid, trichloroacetic acid or scarification in Experiment III, on one side of the skin of the back at various intervals before the injection of urethane. The tumour yield on the treated side, as compared to the untreated side, was significantly augmented when the urethane was injected 24 hours later, reached a maximum at an interval of two or three days and was declining by the fifth or sixth day. The overall pattern of the effect therefore appears to be similar to the augmenting influence of a preliminary application of croton oil reported by Pound and Bell (1962) and Pound (1963).

In the present work the changes in the skin produced by the irritants, as observed macroscopically, were graded in descending order of severity: (*a*) acetic acid, mechanical abrasion, cantharidin and iodoacetic acid; (*b*) trichloroacetic acid and xylene; (*c*) turpentine. This grading appears to agree tolerably with the ranking of the maximum augmenting effects on the tumour yields, namely xylene, acetic acid and turpentine in Experiment II and cantharidin, iodoacetic acid, scarification and trichloroacetic acid in Experiment III. The augmenting influence of a 0·5 per cent solution of croton oil in acetone was insignificant by the third day (Pound and Bell, 1962). If a larger dose of croton oil (0·7 per cent in acetone) is applied, the skin changes are more severe and comparable with the effect of the irritants used in the present work and the augmenting influence then persists for about five days (Pound, unpublished data).

The above features all point to the conclusion that the local augmenting effect is associated with the local phenomena produced by the "irritants" and is related to the severity of them irrespective of the means used which probably act by different biochemical mechanisms. More generally, it appears likely that any means that produce these phenomena at an appropriate time would augment the

tumour yield. The common feature of the effective preliminary treatments is to produce inflammation, almost at once and after a delay of some hours cellular proliferation leading to scaling hyperkeratosis in the skin over the next four or five days.

Increased availability of urethane to the skin, consequent upon vascular dilatation, does not appear to be the significant factor. Thus the urethane was injected subcutaneously between the scapulae and might be expected to produce a local concentration in the skin of the fore half of the back and so increase the tumour yield in this area. However the tumours were randomly distributed over the fore and rear halves of the back as was found similarly by Pound (1963). The augmenting effect is therefore probably bound up with cellular proliferation in the skin, with the implication that dividing cells may be susceptible to the initiating action of urethane.

The irritants used in this work were selected because, in addition to the production of inflammation and cellular proliferation in the skin by different mechanisms, other relevant properties of them were known. Trichloroacetic acid, acetic acid, iodoacetic acid and turpentine did not influence the tumour yield when applied to mouse skin alternately with tar (Berenblum, 1935). Turpentine and xylene failed to influence the tumour yield when applied together with or alternately with benzopyrene and were not carcinogenic when applied alone (Berenblum, 1941). Iodoacetic acid was found to be a mild promoting agent after initiating treatment of mouse skin with 9,10-dimethyl-1,2-benzanthracene while acetic acid, turpentine and cantharidin had no promoting effect and are presumably not carcinogenic (Gwynn and Salaman, 1953). However, in the case of initiation by urethane, the possibility that materials other than croton oil might exert promoting activity does not appear to have been investigated. With this limitation it is clear from the present experiments that the augmenting effect is not related to the promoting capacity of any material nor to any weak carcinogenic property as might be suggested in the case of croton oil. Cantharidin had an anti-carcinogenic effect when applied alternately with repeated applications of tar (Berenblum, 1935) but no such property is manifest in the present experiment in which the converse effect was found on initiation by urethane.

It is of interest that the local application of the irritants led to variation of the tumour yields on the untreated side. The yields on the untreated sides were increased by cantharidin, iodoacetic acid and xylene in descending order. It must be assumed that random variations and genetic variations in the susceptibility of the mice to develop tumours in this experimental system are eliminated by random selection of the mice into groups, although the latter would be one source of an overall correlation between the tumour yields on the two sides in individual mice within the groups. The second factor to produce this influence in the untreated side would be spread of the preliminary treatment to the untreated side. Reasons have been advanced above for believing that this did not occur. Further evidence against this as a cause of the variation is the fact that there was no significant variation between days as on the sides locally treated. Thirdly, the remaining explanation is that cantharidin, iodoacetic acid or xylene exerted a systemic effect that influenced the tumour yield and it is relevant that these substances were those that showed evidence of toxicity to the mice. It is clear that the source of the variation of the tumours on the untreated sides is a matter for further investigation.

Lastly the concentration of tumours along the lines of scarring produced by acetic acid should receive some comment. This result is best interpreted only as a concentration of tumours in an area where ulceration has been severe with resulting increased proliferation in the tissues.

SUMMARY

1. Mice were injected with urethane as a tumour initiator and subsequently painted once each week for twenty weeks with croton oil over the whole area of the skin of the back as a promoting treatment.

2. Groups of these mice were either scarified, or given a single preliminary application of acetic acid, trichloroacetic acid, iodoacetic acid, xylene, turpentine or cantharidin on the right side of the skin of the back at various intervals before the injection of urethane. The preliminary treatments, as judged by the epidermal hyperplasia and inflammation produced, were confined to the treated area. Other groups of mice were treated with acetone to the skin of the back before the administration of the urethane, the acetone produced no gross change in the skin.

3. Prior application of acetone did not influence the tumour yield.

4. The yield of tumours in the mice treated by scarification or the various chemicals was greater on the treated side than on the untreated side. The augmenting influence was significant if the interval between the preliminary application and injection of urethane was 24 hours, reach a maximum at an interval of two or three days and declined by the fifth or sixth day.

5. The local augmenting effect of the several treatments appears to be associated with, and related to the severity of, the local phenomena produced in the skin.

6. In addition to the local augmenting influence, preliminary treatment of the skin with cantharidin, iodoacetic acid or xylene appeared to have a general effect that influenced the tumour yields.

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FURTHER OBSERVATIONS CONCERNING THE INFLUENCE OF PRELIMINARY STIMULATION BY CROTON OIL AND ACETIC ACID ON THE INITIATION OF SKIN TUMOURS IN MICE BY URETHANE

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THE number of skin tumours developed in mice that had been given a standard tumour producing treatment, an injection of urethane followed by an application of croton oil once a week for twenty weeks, was increased if the animals were given a preliminary application of croton oil to the skin a short interval before the injection of urethane (Pound and Bell, 1962). The augmented tumour yield was localized to the area given the preliminary treatment (Pound, 1963). A similar augmenting effect was obtained by preliminary treatment of the skin with any other means which, like croton oil, produced inflammation and cellular proliferation in the skin (Pound and Withers, 1963) and appeared to increase with increasing severity of the local tissue response to the preliminary treatment.

The present experiments were carried out to supply more detail of these aspects of the work, to examine the influence of prior stimulation on tumour formation after local application of urethane to the skin, and to determine if the augmented tumour yield was a permanent characteristic of the mice after initiation with urethane, that is, if it was still found when the promoting treatment with croton oil was delayed.

MATERIALS AND METHODS

Mice

Male mice of the "Hall" strain bred in this department (Pound, 1962*a*) were used. The animals weighed 22–28 g. at the beginning of the experiments and were of more uniform weight in Experiments I and II, namely 23–25 g. They were accommodated in stainless steel compartments each holding originally ten mice. Bedding was provided as a layer of coarse saw-dust that was changed weekly. The mouse room was air conditioned at about 22° C.

The animals were fed standard diets used in previous work; diet as in Pound and Bell (1962) for Experiments III, IV, and V, diet as in Pound and Withers (1963) for Experiments I and II. The diet and water were provided in excess of the animals' needs.

Chemicals

Urethane (ethyl carbamate), British Drug Houses, Laboratory Reagent grade. Acetone, Univar, Analytical Reagent grade. Croton oil, Stafford Allen and Sons, London. Acetic acid, Osta Chemical Company, Analytical Reagent grade.

Urethane by injection was given as a solution in isotonic saline containing 25 mg. per 0·5 ml., sterilized by Seitz filtration. Injections were made subcutaneously between the scapulae.

EXPERIMENTS

Mice in randomly selected groups were given a preliminary treatment of the skin with either croton oil or acetic acid at varying intervals before a tumour producing treatment with urethane. The hair of the back was clipped close to the skin with electric clippers before each application of croton oil, care being taken to avoid injury to the skin which would seem likely to influence the tumour yield in view of previous work from this department (Pound and Withers, 1963).

The tumour producing treatment consisted of a single administration of urethane, by injection of 25 mg. in Experiments I, II, and V, or by local application to the skin of a 25% (w/v) solution in acetone in Experiments III and IV. The animals of each experiment were all treated with urethane on the same day between 2 p.m. and 3 p.m. From one week after the administration of urethane, or after a delay of thirty weeks in experiment V, the animals were given an application of approximately 0·25 ml. of a 0·5% solution of croton oil in acetone to the whole area of the skin of the back once each seventh day for twenty weeks.

The number of tumours of the skin in each mouse was counted once each week before applying the croton oil in Experiment II, but in most experiments the numbers of tumours were counted only once two weeks after the last application of croton oil.

Experiment I

Twenty-two groups of 40 mice were constituted at random. The mice of Groups 1 to 17 were given a preliminary application of approximately 0·25 ml. of a 0·5% solution of croton oil in acetone to the whole area of the skin of the back at 0, 3, 6, 9, 12, 18, 24 hours, 3, 4, 5, 8, 9, 10, 11, 12 or 14 days respectively before injection of 25 mg. urethane. The animals of Groups 18, 19, 20, and 21 were given a preliminary application of 0·25 ml. of 0·5% solution of croton oil in acetone to the right side of the skin of the back 0, 1, 3, and 10 days respectively before the injection of urethane. The mice of control Group 22 had no treatment with urethane but were given the standard twenty applications of croton oil alone.

Because of the number of mice involved the groups were divided equally to form two lots, one lot being given each of the twenty weekly applications of croton oil one day later than the other.

Experiment II

Fourteen groups of 40 mice were constituted at random. The mice of Groups 1, 2, etc. to 12 were given a preliminary application of approximately 0·25 ml. of a 0·7% solution of croton oil in acetone to the whole area of the skin of the back at 0, 3, 6, 9, 12, 15, 18, 24 hours, 2, 3, 4 or 5 days respectively before the injection of 25 mg. urethane. The mice of control Groups 13 and 14 were given only the standard twenty weekly applications of croton oil or the injection of urethane alone respectively.

The animals of Experiment II were randomized with those of Experiment I and injected with urethane one day later.

Experiment III

In eight groups of 20, mice were administered the urethane by local application of approximately 0·25 ml. of a 25% (w/v) solution in acetone, about 64 mg.

urethane, to the whole area of the skin of the back. At 5, 3, 2 days, 24, 18, 12, 6, and 0 hours before the treatment with urethane the animals in the respective groups were given a preliminary treatment to the skin of the right side of the back with approximately 0·25 ml. of 0·5% solution of croton oil in acetone.

Experiment IV

This was executed in the same manner as Experiment III except that a 20% solution (v/v) of acetic acid in acetone was used instead of the solution of croton oil for the preliminary treatment.

Experiment V

Four groups of 40, or, in one instance, 60 mice were randomly selected. The mice of two groups were injected with 25 mg. urethane. The mice were painted with croton oil once weekly for 20 weeks, those in one group from the seventh day after the injection and those in the other group (60 mice) after a delay of 210 days (30 weeks) from the day of injection. The mice in the remaining two groups were treated similarly after an application of 0·25 ml. of 20% acetic acid solution in acetone to the whole area of the skin of the back 18 hours before the injection of urethane.

RESULTS

Thirty-five of the 40 mice injected with urethane alone (Experiment II, Group 14) survived but none developed a papilloma on the skin of the back. Out of the 80 mice treated with 20 weekly applications of croton oil alone (Experiment I, Group 22 and Experiment II, Group 13), 74 survived and five of these animals developed a total of six papillomata. Since the incidence of spontaneous papillomata in these mice is probably less than one in a thousand, this is a significant number ($P < 10^{-6}$) as regards the minor carcinogenic effect of croton oil but is a negligible fraction of the number of tumours obtained when the animals had been administered urethane by injection (Tables I, II, and V) or by local application to the skin (Table IV). These control groups therefore are not considered any further.

The proportion of surviving mice in Groups 2 and 18 of Experiment I (Table I) was less than in the other groups of Experiments I and II but this was due to a "rogue" mouse in one tin of each of these groups killing off his fellows early in the experiment and was therefore unrelated to the experiment. Even so, the variation in the survival rates between the groups was not statistically significant so that it can be ignored as a factor that might have influenced the tumour yields between the groups in the discussion that follows. However, this is not the case for the results of Experiment V, as dealt with below.

Tables I, II, IV, and V set out the number of surviving mice, the number of mice with tumours and the number of tumours in the survivors, 2 weeks after the last of the twenty weekly applications of croton oil following the administration of the urethane. The results of Experiments I, III, and IV are dissected to show firstly the number of mice with tumours on the right side, mid-line and left side, and secondly the number of tumours on the right side, mid-line and left side as described previously (Pound and Withers, 1963) since, in these experiments, it was necessary to compare corresponding areas of the two sides when these differed

in the preliminary treatment and because mid-line tumours can not be assigned to any side.

The effect of preliminary application of croton oil before injection of urethane. Experiments I and II

It is clear from the results of Experiments I and II (Tables I and II respectively and Fig. 1) that the number of skin tumours developed in the surviving mice varied as the time interval between the preliminary application of croton oil and the injection of urethane was increased.

After a preliminary treatment with a 0·5% solution of croton oil in acetone, Groups 1 to 17 of Experiment I (Table I and Fig. 1), the number of tumours per

TABLE I.—*The Influence of a Preliminary Application of 0·5% Croton Oil Solution in Acetone at a Varying Interval before an Injection of 25 mg. Urethane on the Tumour Yields (Experiment I)*

		Survivors								
			Mice with tumours				Number of tumours			
Group	Interval before Injection of Urethane	Number of mice	on left side	in mid-line	on right side	Total	on left side	in mid-line	on right side	Total
1	14 days	35	8	3	9	15	10	3	14	27
2	12 ,,	28	7	1	6	10	9	2	10	21
3	11 ,,	39	9	1	11	14	18	1	20	39
4	10 ,,	34	10	4	11	15	17	5	15	37
5	9 ,,	37	11	1	11	19	18	1	21	40
6	8 ,,	39	11	2	10	16	12	2	13	27
7	5 ,,	37	14	1	14	18	28	1	21	50
8	4 ,,	39	11	2	12	15	15	2	18	35
9	3 ,,	36	14	2	12	19	26	5	31	62
10	24 hours	37	17	8	22	26	61	13	65	139
11	18 ,,	33	21	7	22	26	66	12	74	152
12	15 ,,	36	24	5	23	27	66	12	67	145
13	12 ,,	35	14	4	13	24	35	5	32	72
14	9 ,,	34	15	3	14	25	23	7	29	59
15	6 ,,	38	11	1	12	18	19	2	12	33
16	3 ,,	37	13	2	13	18	16	4	19	39
17	0 ,,	34	7	4	11	13	19	5	15	39
18	10 days	26	5	2	7	—	13	2	9	—
19	3 ,,	36	10	4	12	—	18	4	35	—
20	24 hours	37	12	6	21	—	18	7	67	—
21	0 ,,	36	9	2	11	—	15	2	23	—
22	0 C	36	1	—	2	3	1	—	2	3

Forty mice in each group at beginning of experiment.
C = Mice of Group 22 painted with croton oil promoting treatment only.

surviving mouse did not vary at intervals of 0, 1, 3, 6 hours, was slightly greater at 9 hours, and increased abruptly from 12 hours to a maximum at about 18 hours. As the interval increased further, the augmenting effect declined so that at the fifth day, the tumour yield reached a basal level that was not significantly different from that at the intervals of 0, 3, and 6 hours. A similar rise and fall occurred in the number of tumours per tumour bearing mouse.

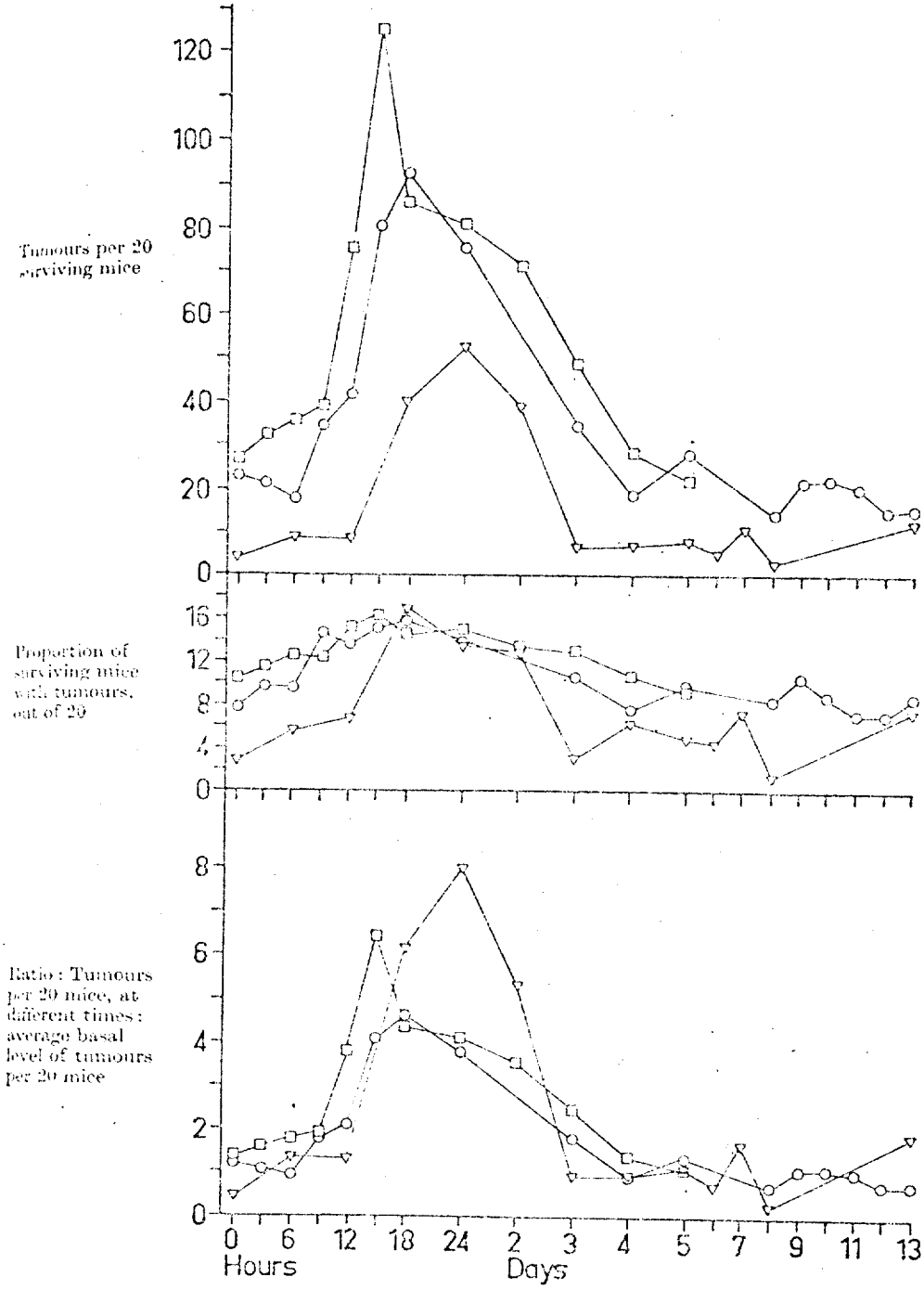

FIG. 1.—The influence of a preliminary application of croton oil at an increasing interval before an injection of urethane on the number of tumours in the surviving mice, on the proportion of surviving mice bearing tumours, and on the ratio of the augmented number of tumours in the surviving mice to the basal level of the number of tumours in the surviving mice.

○ 0·5% solution of croton oil in acetone.
□ 0·7% solution of croton oil in acetone.
▽ 0·5% solution of croton oil in acetone, data of Pound and Bell (1962).

Time Scale is discontinuous at 24 hours and at 5 days.

TABLE II.—*The Influence of a Preliminary Application of 0·7% Croton Oil Solution in Acetone at a Varying Interval before an Injection of 25 mg. Urethane on the Tumour Yields (Experiment II)*

Group	Interval before injection of urethane	Survivors: Number of mice	Survivors: Mice with tumours	Survivors: Number of tumours	Time of appearance of first tumour (weeks)	Mean time of each tumour (weeks)
1	5 days	37	17	40	10	15
2	4 ,,	32	17	43	10	17
3	3 ,,	37	24	90	8	13
4	2 ,,	34	23	121	8	12
5	24 hours	32	24	129	6	12
6	18 ,,	36	26	156	6	13
7	15 ,,	36	29	203	4	13
8	12 ,,	37	28	139	5	12
9	9 ,,	34	21	63	7	15
10	6 ,,	37	23	65	8	12
11	3 ,,	35	20	55	9	16
12	0 ,,	37	19	48	10	16
13	C_1	38	2	3	15*	18*, 20*
14	C_2	35	0	0	—	—

Forty mice in each group at beginning of experiment.
C_1 = Mice of Group 13 painted with croton oil promoting treatment only.
C_2 = Mice of Group 14 injected with urethane only.
* = Times of appearance of the three tumours.

A practically identical pattern of increase in the tumour yields was found after the application of 0·7% croton oil in acetone (Experiment II, Table II and Fig. 1). The number of tumours per surviving mouse and the number of tumours per tumour bearing mouse increased abruptly at an interval of 12 to a maximum at 15 hours and then returned by about the fifth day to a basal level that was not significantly different from that in Experiment I. There appears to be some increase in the number of tumours per surviving mouse at the intervals of 6 and 9 hours.

On comparison of the results of Experiments I and II, statistical analysis (Table III) shows that the increase in the number of tumours per surviving mouse in response to 0·7% croton oil rose more sharply and reached a greater maximum than in response to 0·5% croton oil. However, if the parameter of the number of tumours per tumour bearing mouse was taken the greater increase in the tumour yield was apparent only at the maximum.

The proportion of mice bearing tumours, the third parameter, appeared to follow a similar pattern of variation (Fig. 1), rising and falling with the number of

TABLE III.—*Comparison of Effects of Preliminary Treatments with 0·5% Croton Oil (Experiment I) and 0·7% Croton Oil (Experiment II) on Tumour Yields, Data of Tables I and II Respectively*

Interval group	Tumours per surviving mice	Tumours per tumour bearing mouse
5, 4, and 3 days	$\chi^2 = 3{\cdot}57$, N.S.	$\chi^2 = 0{\cdot}18$, N.S.
24, 18, 15 and 12 hours	$\chi^2 = 12{\cdot}27$, $P < 0{\cdot}001$	$\chi^2 = 17{\cdot}18$, $P < 0{\cdot}001$
9, 6, 3, and 0 hours	$\chi^2 = 8{\cdot}98$, $P < 0{\cdot}01$	$\chi^2 = 3{\cdot}43$, N.S.

For obvious reasons only the groups in each experiment with the intervals shown could be compared; for the purposes of the analysis they were combined in interval groups as shown in the table.

tumours in the surviving mice, but in neither Experiment I nor Experiment II did the variation in this parameter reach a level of statistical significance. This parameter was therefore relatively insensitive to the treatments compared with the number of tumours per tumour bearing mouse, or with the number of tumours per surviving mouse.

It is also evident from the results of Experiment II (Table II), that the first tumours appeared earlier and the mean of the time of appearance of the tumours tended to be earlier in the groups with the larger tumour yields than in the other groups. Relationships of this sort might of course be expected because these two variables are related to each other and also to the tumour yields. The differences in the means recorded are not great and even though possibly significant statistically, the further biological interpretation of such results in this type of experiment (where the development of tumours is truncated at 20 weeks!) is limited, so that these results are not considered further.

In Experiment I, Groups 1 to 17, there was no significant difference between the number of mice with tumours on the right and left sides nor between the number of tumours developed on the two sides, irrespective of the augmenting effect of the preliminary application of croton oil. In Groups 18 to 21, in which the preliminary application of (0·5%) croton oil was made only on the right side of the skin of the back at varying intervals before injection of urethane, there was no significant variation between the numbers of tumours in the surviving mice on the left side. However, there was a significant variation between the number of tumours developed on the right side that was clearly due to the increased number of tumours on this side in the groups given the preliminary application of croton oil 24 hours and 3 days before the administration of urethane. Moreover the increased tumour yield over the yield in the untreated side was of the same order as the increase in the yield in Groups 1 to 17 so that the augmenting effect was due to local phenomena produced in the area that had the preliminary treatment in conformity with previous results (Pound, 1963).

Comparison of results of Experiments I and II with previous results

Since it was an object of the present work to examine the augmenting effect in more detail, the results in Tables I and II are to be compared with those reported earlier (Pound and Bell, 1962). It was then shown that an application of 0·5% croton oil in acetone at various intervals before injection of 25 mg. urethane augmented both the proportion of mice bearing tumours and the number of tumours in the surviving mice at intervals of 18, 24, and 48 hours (Fig. 1). In these earlier results the basal level of the proportion of mice bearing tumours, about 4·7 out of 20 mice, and the number of tumours developed in the surviving mice, about 6·5 per 20 mice, were both lower than in the present work in which the basal levels in both Experiments I and II were about 8·6 out of 20 mice for the proportion of mice bearing tumours and 20 per 20 mice for the number of tumours in the surviving mice.

In the present work, the proportion of surviving mice with tumours was less sensitive than the number of tumours in the surviving mice as a measure of the augmenting effect, and is therefore unsuited to comparison between the two sets of results.

However, the interest is to compare the augmenting effect as such. A comparison of the ratios of the number of tumours in a given number of surviving mice

at any particular interval to the basal level of the number of tumours in the same number of surviving mice in the three experiments might, to some extent, obviate the influence of some of the factors responsible for the different basal levels. These are plotted in Fig. 1. It is evident that the ratios derived from the present results are of the same order as those derived from the results previously reported (Pound and Bell, 1962). The slightly greater effect of 0·7% croton oil as against 0·5% in the present experiments is still evident.

The effects of a preliminary application of croton oil or acetic acid before administration of urethane by local application to the skin

The results of Experiment III, in which the preliminary application of croton oil was made on the right side of the skin of the back at various intervals before the local application of 60 mg. urethane dissolved in acetone to the whole area of the skin of the back, are set out in Table IV. No significant variation was found

TABLE IV.—*The Effect of Preliminary Application of Croton Oil or Acetic Acid before Local Application of Urethane*

Preliminary treatment	Group	Interval between preliminary treatment and application of urethane	Survivors: Number of mice	Mice with tumours: on left side	Mice with tumours: in mid-line	Mice with tumours: on right side	Number of tumours: on left side	Number of tumours: in mid-line	Number of tumours: on right side
Experiment III									
Croton Oil	1	5 days	17	2	1	2	2	1	3
	2	3 ,,	18	3	0	3	3	0	7
	3	2 ,,	13	2	1	6	3	1	14
	4	24 hours	18	3	1	7	4	4	19
	5	18 ,,	17	2	1	6	3	3	17
	6	12 ,,	20	3	0	2	5	0	5
	7	6 ,,	19	2	1	2	2	1	4
	8	0 ,,	18	2	0	2	3	0	2
Experiment IV									
Acetic acid	1	5 days	16	2	0	4	3	0	4
	2	3 ,,	17	2	1	5	2	1	9
	3	2 ,,	14	3	1	7	3	2	14
	4	24 hours	18	3	0	7	6	4	24
	5	18 ,,	16	3	1	4	4	1	12
	6	12 ,,	18	2	0	6	3	0	9
	7	6 ,,	16	1	0	2	1	0	2
	8	0 ,,	19	2	0	0	2	0	0

Twenty mice in each group at beginning of experiment.

between the number of tumours on the left side of the back, but there was a significant variation between intervals in the number of tumours on the right side that was clearly due to increased tumour yields at the intervals of 18, 24, and 48 hours between the preliminary application of croton oil and the application of urethane.

When acetic acid instead of croton oil solution was applied to the right side of the skin of the back at various intervals before the application of urethane to both sides (Experiment IV, Table IV), a similar augmented tumour yield was found on the right side at the intervals of 18, 24, and 48 hours.

It is therefore clear that a preliminary application of croton oil or acetic acid before local application of urethane to the skin had an augmenting effect on the tumour yields similar to that which occurred when the urethane was administered systemically. The similarity is both to the variation with the intervals between the applications of croton oil and the urethane, and to the actual relative increase in the tumour yields in the treated mice.

The mice painted with an acetone solution of urethane became drowsy, to the degree that might be expected had they ingested about 12 mg. urethane, so that considerable absorption of urethane must have occurred. Were this not the case, results such as these would be strong evidence against the view that the augmenting effect was due to a dosage phenomenon consequent upon vascular dilatation.

Persistence of the augmenting effect on the initiation of tumours

The tumour yields in mice given no preliminary application or a preliminary application of acetic acid 18 hours before injection of urethane, and in which the promoting treatment of 20 weekly applications of croton oil was commenced after 7 days or after an interval of 210 days, Experiment V, are set out in Table V.

TABLE V.—*Effect of Delay between "Initiating" and "Promoting" Treatments on Tumour Yields (Experiment V)*

Group	Preliminary treatment	Mice: Number at outset	Mice: Number commencing promotion	Delay in commencing promotion (weeks)	Survivors: Number of mice	Survivors: Mice with tumours	Survivors: Number of tumours
1	None	40	40	1	37 (1) (3*)	15	29
2	,,	60	49 (5)	30	39 (5) (4*)	7	14
3	Acetic acid	40	40	1	38 (1) (1*)	33	165
4	,, ,,	40	33 (5)	30	24 (6) (2*)	22	66

Figures in parentheses are the number of mice dead with evidence of lymphoma at the time indicated. Asterisked figures in parentheses are the number of surviving mice with clinical evidence of lymphoma at the time of counting tumours.

Groups 1 and 3 showed the expected increased tumour yield produced by the preliminary application of acetic acid. The results of Groups 2 and 4 showed that the increased yield persisted even though the promoting treatment was delayed 30 weeks, although the tumour yields in both Groups 2 and 4 were significantly less than in the corresponding Groups 1 and 3 respectively. However, the interpretation must be influenced by the facts that the mice had reached an age of about 56 weeks and many of them were in poor condition. There was a significant increasing proportion of mice with enlarged lymph nodes, enlarged spleens, and leukaemic blood pictures. These factors are reflected in the significantly lower survival rates. All these factors would necessitate rigid experimental controls to elucidate this point.

DISCUSSION

Ethyl carbamate by itself, when applied locally to the skin (Graffi *et al.*, 1953; Salaman and Roe, 1953; Roe and Salaman, 1954; Berenblum and Haran, 1955), administered by mouth (Haran and Berenblum, 1956; Berenblum and Haran-Ghera, 1957) or injected into mice (Berenblum and Haran-Ghera, 1957) does not

lead to the formation of tumours of the skin except when followed by repeated applications of a "promoting" agent such as croton oil. Urethane is therefore often regarded as a "pure initiator" in the terms of the two stage hypothesis (Salaman, 1958). Ideally for the two stage hypothesis, the promoting agent itself should not produce any tumours. However, croton oil appears to exert a mild carcinogenic effect after prolonged treatment to the skin (Roe, 1956; Boutwell, Bosch and Rusch, 1957) as also seen in the control mice of the present experiments. On the other hand, Lindsay (1956) found that applications of urethane alone to the skin of the susceptible NYZ strain of mice produced some papillomata. In the mice of the strain used in this laboratory, out of 1000 mice injected with urethane eight papillomata of the skin have been found in 626 that survived for 13 months, whereas the normal incidence of papillomata in these mice is low, less than one in a thousand mice reaching this age (Pound, unpublished data). The two stage hypothesis, therefore, in the case of urethane as in the case of carcinogenic hydrocarbons, should be regarded only as an incomplete working hypothesis. Nevertheless, the administration of urethane by any route produces a change in the skin of mice such that it is predisposed to the development of tumours when subsequently treated with croton oil. Since the number of tumours obtained on the combined treatment is much greater than that produced by croton oil alone, and many more times greater than the number of tumours produced by urethane alone, it is improbable that this results from the simple addition of the effects of two weak carcinogens. The nature of the change in the skin is not known. The author has interpreted this change in the most general terms as representing the more or less permanent production of potential tumour forming foci in the tissue; this does not imply any subsidiary hypothesis of an all or nothing effect, a change confined to single individual cells, or, since cells are clearly affected in the change, of involvement of only one particular type of cell.

The experiments reported in this paper show that a preliminary local application of croton oil to the skin a short interval before the injection of urethane increases the tumour yield in the treated area and, with the previous results (Pound and Bell, 1962; Pound, 1963), now allow a clear description of the variation of the tumour yield with length of the interval. With a preliminary application of 0·5% croton oil in acetone there is a negligible change until an interval of 9 hours; from 12 hours the tumour yield increases abruptly to a maximum at about 18 hours, after which it declines to the basal level by the fifth day. Using 0·7% croton oil, the variation with length of the interval follows the same pattern; there is still the abrupt increase at 12 hours and the yield returns to the same basal level as with 0·5% croton oil, but the tumour yield increases more rapidly and rises to a somewhat higher level. When urethane is applied to the skin, the previous application of either croton oil or acetic acid augments the tumour yield to an extent that varies with the length of the intervening interval in a similar manner. The augmenting effect of croton oil was also found after administration of urethane in the diet (Pound, 1962*a*) and is therefore independent of the route of administration of the urethane.

Pound and Withers (1963) reported that preliminary treatment of the skin by scarification or with a variety of chemicals which, like croton oil, produced inflammation or cellular proliferation in the skin also produced an augmented tumour yield in the treated area that appeared to follow a similar variation with the length

of interval between preliminary treatment and the injection of urethane, although the evidence then presented was incomplete in the latter respect. It is now clear from the results of the present experiments in which a preliminary local treatment with acetic acid was used, that the variation with this interval indeed follows practically the same pattern as with a preliminary application of croton oil. It may be deduced that, as a general rule, the same pattern of variation would be found after treatment of the skin with any agent that produced similar local effects, although some variation in detail might be expected in particular instances.

Furthermore, it has been demonstrated that the change in the tissues induced by urethane persists if the commencement of the "promoting" treatment with croton oil is delayed for as long as 30 weeks, although both the basal level of tumours produced in the absence of any preliminary treatment and the augmented tumour yield produced by a preliminary application of acetic acid at 24 hours before the injection of urethane decline significantly. The elaborate control series of animals that would be necessary to ascertain the sources of this variation is lacking and it cannot be determined if the decrease results from a change in the character or the number of potential tumour forming foci produced by the urethane or whether it is due to other factors. Persistence of the change in the skin produced by urethane was reported by Berenblum and Haran-Ghera (1957) after a delay of 59 days. Roe and Salaman (1954) had found that when the delay was 24 weeks, the tumour yields decreased by as much as 50% but this was considered only doubtfully significant because of the poor condition of the mice. However, the important facts are that a substantial proportion of the capacity of the skin to produce tumours, induced by urethane, persists and that the rates of decline in both the mice with the usual tumour yield and mice with the augmented tumour yield are similar.

The above characteristics of the augmented tumour yields are, of course, similar to those normally obtained after the action of urethane alone and suggest that the basic range of change produced is not affected by the preliminary stimulation. Also there is no evidence from the results reported in this paper, from Pound and Bell (1962) or from Pound and Withers (1963), that the time of appearance of the tumours is significantly accelerated, although this aspect might warrant specific critical examination. Further, the structure of the lesions and the manner of growth do not appear to differ. It may be inferred therefore that the general biological characteristics of the change remain unaltered when augmented by the preliminary treatment. The augmented yield, which is the measure of this change, may thus be due to an increased number of potential tumour forming foci that can respond to a given promoting treatment rather than due to an intensification of the change in a similar number of foci.

The basal level of the tumour yields in the comparable experiments of the present work is higher than that obtained in the experiment reported previously (Pound and Bell, 1962). Since the yield of tumours in the controls treated with croton oil alone was also higher, this may be associated wholly or partly with factors affecting the "promoting" treatment and the different batch of croton oil used. It may also be noted that the diet was changed but probably other factors are also at work. Nevertheless, considered as the ratio of the augmented tumour yield to the basal yield, the effect of the preliminary application of croton oil is of similar order in both experiments. The relatively greater effect in the earlier experiment is possibly to be ascribed to a greater potency of the sample of croton

oil used to produce epithelial hyperplasia. The probability that the number of tumours produced by urethane can be increased in a certain ratio by the preliminary treatment with croton oil is therefore independent of the promoting treatment, that is the preliminary treatment acts only on the "initiation" stage by urethane.

The augmented tumour yields brought about by preliminary scarification and the various chemicals used by Pound and Withers (1963) increased with the increasing severity of the local changes they each produced. This trend is further evidenced by the greater effect of a 0·7% as against a 0·5% solution of croton oil in the present experiments. The stronger solution of croton oil produced more severe inflammation and hyperplasia of the epidermis, although the difference was not great and in neither case, with the particular sample of croton oil used, were the changes as gross as those that followed application of acetic acid. It is of considerable interest that these materials and acetic acid in this work are neither carcinogenic nor "promoting" agents and thus differ from croton oil. The augmenting effect is therefore not associated with these properties of croton oil but appears to be related to the common properties of all these materials of producing inflammation and cellular proliferation in the skin. Some additional reasons were put forward (Pound and Withers, 1963) to support the original opinion (Pound, 1962*a*; Pound and Bell, 1962; Pound, 1962*b*) that the augmented tumour yields, and, by implication, the increased number of potential tumour forming foci of which they are an index, are the result of an increased susceptibility of proliferating tissues to the action of urethane rather than due to a dose phenomenon consequent upon the vascular dilatation of inflammation.

Whether vascular dilatation as such could offer an explanation of this effect is open to question; even if it is not readily amenable to rigorous experimental investigation since circumstances that produce it would seem likely to produce cellular proliferation in the tissue (Bullough, 1962; Bullough and Laurence, 1964). Urethane is a highly diffusable substance of small molecular weight and after injection appears to be rapidly and uniformly distributed throughout the tissues of the body (Bryan, Skipper and White, 1949; Mitchell *et al.*, 1949; Skipper *et al.*, 1951; Boyland and Rhoden, 1949; Berenblum *et al.*, 1958) so that it appears likely that cells of the injected animal would be exposed to a uniform concentration of urethane that would not be materially altered by vascular dilatation or oedema. Unless the cells themselves selectively took up the urethane the increased availability of the drug should not be of great significance. Certain cells, such as sea urchin eggs (Cornman, Skipper and Mitchell, 1951) readily take up this material, but it can be removed easily by washing. That urethane is taken into mammalian cells is obvious from the variety of cytological effects it produces in the tissue and other properties (for review see Cornman, 1954). However, the available evidence is not convincing that it is selectively taken up in amounts greater than can be expected from diffusion (Skipper *et al.*, 1951; Berenblum *et al.*, 1958). It may be significant that dividing sea urchin eggs rapidly accumulated it in amounts exceeding the surrounding concentration (Cornman *et al.*, 1951). Berenblum and Haran-Ghera (1957) found that doses of 16 and 64 mg. of urethane administered by stomach tube to mice gave, as calculated from their data, tumour yields of 18 tumours per 24 surviving mice and 120 tumours per 24 surviving mice respectively, and in one experiment in this laboratory an increase in the dose of urethane by subcutaneous injection from

25 mg. to 50 mg. increased the tumour yield from 18 per 36 mice to 48 per 35 mice (Pound, unpublished data). Thus it would seem reasonable to expect that the production of the four-fold or greater increase in the tumour yield produced by the preliminary treatments of the skin with croton oil or other chemicals would require the development of a local concentration of urethane about equal to that obtained by a four-fold increase in the dose of urethane and it seems unlikely that this would be attained by vascular dilatation.

However, long chains of reasoning do not constitute scientific progress. Histological studies (Pound, as yet unpublished) in fact demonstrate that the vascular dilatation after an application of croton oil develops more rapidly and reaches a peak earlier than the beginning of the augmented tumour yield. On the other hand, there is clear evidence from many fields that following cellular damage to a field of tissue produced by any means there occurs a burst of mitotic activity preceded by a delay of 12 to 24 hours or even more (Bullough, 1962; Bullough and Laurence, 1964). Studies in this laboratory show that, after an application of 0·25 ml. of a 0·5% solution of croton oil in acetone, a burst of mitotic activity commences abruptly about 15 hours later to reach a maximum in about 12 hours after this. This does not correlate well with the pattern of the augmented tumour yields described above. On the other hand, a similar burst of replication of DNA, as measured by tritiated thymidine labelling of epidermal nuclei, commences abruptly some hours earlier than the burst of mitosis and in fact follows reasonably well the pattern of the increase of the tumour yields. These observations would appear to indicate that urethane acts on cells at a stage of the cycle of cell division corresponding to the phase of synthesis of DNA. However it seems improbable that this is a direct effect.

It may be recalled that Mottram (1944, 1945) showed that a preliminary application of croton oil to one flank of mice locally augmented the tumour yield produced by an application of the carcinogenic hydrocarbon benzopyrene to both flanks, and associated the increase with the abundant cellular proliferation that resulted. These results have not been repeated and the work discounted because multiple applications of croton oil before the hydrocarbon failed to augment the tumour yield. Recently Tannenbaum, Vesselinovitch and Silverstone (1964) have claimed such an augmenting effect after many trials of this latter character, but it is doubtful for various reasons, amongst them those mentioned by Pound and Bell (1962) and others, whether in this type of experiment the slightly augmented tumour yield is in fact due to the phenomenon dealt with above in this paper. However, it will be shown (Pound, as yet unpublished) that a preliminary application of croton oil or acetic acid does in fact augment the tumour yield after application of carcinogenic hydrocarbons and moreover that augmented tumour yield bears the same relationship or an analogous one to the interval between the preliminary and initiating treatments as is found in the case of urethane.

SUMMARY

Mice were given a standard tumour producing treatment consisting of an administration of urethane followed by twenty weekly applications of croton oil as a promoting agent.

A preliminary application of croton oil a short interval before administration of the urethane increased the tumour yields in the treated areas. There was

little effect at an interval of 9 hours or less. At 12 hours the tumour yield increased abruptly, it reached a maximum at an interval of 15 to 18 hours, and returned to normal at about 5 days. A similar increase in the tumour yield was produced by preliminary application of acetic acid.

The relative increase in the tumour yields was similar if the urethane was administered by injection or by application to the skin, and appeared to be independent of the efficacy of the promoting treatment. It was of similar order if the promoting treatment was delayed for 30 weeks, although the actual tumour yields decreased.

Reasons are put forward to suggest that the augmented tumour yield is the result of an increased number of potential tumour forming foci rather than an intensification of the change in the same number of foci, and for the view that the augmenting effect is associated with production of cellular proliferation in the tissue, in particular with the replication of DNA.

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THE INITIATION OF SKIN TUMOURS IN MICE BY HOMOLOGUES AND N-SUBSTITUTED DERIVATIVES OF ETHYL CARBAMATE

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Summary. Several homologues and N-substituted derivatives of ethyl carbamate have been examined for tumour-initiating properties by injection into mice followed by promoting treatment with croton oil. The LD_{50} of the compounds was also determined. The tumour-initiating potencies were ranked in descending order ethyl carbamate, ethyl N-methylcarbamate, ethyl N-ethylcarbamate butyl carbamate, n-propyl and isopropyl carbamates with doubtful effect; and ethyl N-n-propylcarbamate, ethyl N,N-dimethylcarbamate, ethyl N,N-diethylcarbamate and methyl carbamate without significant effect. The LD_{50} of homologous series decreased exponentially with increasing molecular weight. It was deduced that the tumour-initiating properties do not vary with the toxic properties of the carbamates nor with their molecular weight but appear to depend on structural features of the molecule, namely the nature of the ester group, the presence of a free hydrogen in the amide position and the nature of a single substituent in the amide position.

INTRODUCTION.

Skin tumours in mice are not usually produced by the administration of ethyl carbamate alone, but appear only after the skin is subsequently painted repeatedly with a promoting agent such as croton oil (Salaman and Roe, 1953; Haran and Berenblum, 1956), that is, in terms of the two stage hypothesis (Salaman, 1958), it effects the initiation stage of skin carcinogenesis. This hypothesis postulates that the initiating agent converts a few normal epithelial cells into dormant tumour cells which require a further stimulation by a promoting agent for development into progressively growing tumours. Agents which produce inflammation and cellular proliferation in the skin increase the number of tumours produced when applied a short interval before the administration of urethane, and the view has been put forward that proliferating cells are more susceptible, or indeed those responsive, to the initiating action of urethane (Pound and Bell, 1962; Pound and Withers, 1963; Pound, 1966).

The potency of carbamates to initiate skin tumours appears to be restricted to the ethyl ester or a few related compounds (Berenblum, Ben-Ishai, Haran-Ghera, Lapidot, Simon and Trainin, 1959) which are regarded as less potent. The present experiments were carried out primarily to examine the influence of stimulation of the skin by acetic acid on the number of tumours initiated by the subsequent administration of some homologues and N-substituted derivatives of ethyl carbamate. The toxicities of the carbamates on subcutaneous injection were determined.

Materials and Methods.

Mice.

Random bred male mice of the strain "Hall" (Pound, 1962) were used. The animals were divided into groups by random selection and were housed in stainless steel tins with a bed of coarse sawdust that was changed weekly. They were fed a standard diet used in previous work (Pound and Withers, 1963) supplied with water in excess of the animals' needs. The mouse room was air conditioned at 22°.

The animals, of an average age of about 7 weeks, weighed 25 ± 1 g. at the beginning of the experiments. The hair of the skin of the back was clipped with electric clippers an hour or two before applications to the skin.

Chemicals.

Ethyl carbamate, British Drug Houses, analytical reagent. Methyl carbamate, isopropyl carbamate, n-butylcarbamate, ethyl N-methylcarbamate, ethyl N-ethylcarbamate and ethyl N-n-propylcarbamate, K & K Laboratories Inc., New York. n-Propyl carbamate, Eastman Organic Chemicals, Rochester, New York. Acetone, Univar, analytical reagent. The carbamates were used without further purification.

Ethyl N,N-dimethylcarbamate and ethyl N,N-diethylcarbamate were prepared by the reaction between equivalent amounts of the corresponding secondary amines and ethyl chlorocarbonate in the presence of an equivalent amount of 3 N NaOH in water. The products were extracted with ether and the esters recovered by distillation at atmospheric pressure using a short column. The product boiling between 144 and 146·5° was collected as ethyl N,N-dimethylcarbamate (Pollock and Stevens, 1965, Ethyl N,N-dimethylcarbamate, B.P. 147°); and the product boiling between 168 and 170° was collected as ethyl N,N-diethylcarbamate (Pollock and Stevens, 1965, Ethyl N,N-diethylcarbamate, B.P. 167-169°). The yields were 76% and 86% of theoretical respectively.

Croton oil was prepared in the department by extraction of the seed of *Croton tiglium* (L.) with methanol.

For use the carbamates were made up with saline to a volume of 0·5 ml. per dose. The solid carbamates were kept in solution by warming when necessary. When the liquid carbamates were not completely soluble 1% serum albumin was included in the solution, and reasonably stable emulsions then formed on shaking.

Toxicity of carbamates.

After preliminary tests to determine the approximate lethal doses, groups of ten mice weighing 25 ± 0·5 g. were injected with logarithmically graded doses of each carbamate to give four groups in the range 1-10 deaths. The number of mice dead and the depth of narcosis of survivors was recorded at 2 hr., 6 hr. and 24 hr. after the injections. Depth of narcosis was graded, 18 the mice could be pushed over without resistance; 2, mice were sleeping but could be stimulated to move about; 3, light anaesthesia; and 4, deep anaesthesia.

An estimate of LD_{50} was calculated by the method of Weil (1952). Graphs of the dose against number of mice dead on logarithmic-probability paper fell on straight lines which were approximately parallel for each carbamate.

A few mice died after 24 hr. in the groups injected with ethyl *N,N*-dimethylcarbamate and ethyl *N,N*-diethylcarbamate, but the LD_{50} at 3 days was reduced only to 0·98 and 0·65 respectively, i.e. not significantly (Table 1). Fewer mice died after 24 hr. following injections of the other compounds.

TABLE 1.

Toxicity of carbamates for mice by subcutaneous injection.

Carbamate	LD_{50} g. per kilo — Hrs. after injection: 2	6	24	Dose LD_{50}	Depth of narcosis* Hrs. after injection: 2	6	24
Methyl	>8·3	6·1	4·45	1·0	3	3	1
			3·76—5·16	0·8	2	2	—
Ethyl	3·0	2·5	2·23	1·0	4	3	1
			2·15—2·39	0·8	4	2	—
				0·5	4	1	—
n-Propyl	1·39	1·30	1·30	1·0	4	3	1
			1·20—1·39	0·8	4	3	1
				0·5	3	2	—
iso-propyl	1·92	1·58	1·28	1·0	4	1	2
			1·12—1·47	0·8	4	4	1
				0·5	3	3	—
n-Butyl	0·54	0·54	0·54	1·0	4	1	—
			0·50—0·59	0·8	3	—	—
Ethyl *N*-methyl	>2·0	1·47	1·36	1·0	4	4	1
			1·24—1·48	0·8	4	3	1
				0·5	3	2	1
Ethyl *N*-ethyl	0·86	0·86	0·86	1·0	3	3	—
			0·74—1·00	0·8	2	1	—
Ethyl *N*-n-propyl	0·59	0·54	0·54	1·0	3	2	—
			0·48—0·62	0·8	2	1	—
Ethyl *N,N*-dimethyl	1·13	1·09	1·05	1·0	3†	1†	1†
			0·97—1·16	0·8	1†	1†	—
Ethyl *N,N*-diethyl	0·80	0·77	0·68	1·0	2†	1†	1†
			0·61—0·77	0·8	1†	1†	—

LD_{50} mean values stated, italicised figures are the 95% confidence limits.

* depth of narcosis refers to surviving mice at each time.

† prolonged lethargic state clinically different from narcosis of other compounds, see text.

0·8 LD_{50} can be expected to cause death of 1 - 2 mice out of 10 injected.

0·5 LD_{50} will cause no deaths.

Tumour initiation tests.

Experiment I. Nine random groups of 40 mice were given an application of 0·25 ml. of a 2% solution of acetic acid in acetone to the right side of the skin of the back. Eighteen hr. later the mice of groups 1 to 8 were injected in turn with one of the carbamates in the doses indicated in Table 2; one group was kept as control. From 2 weeks later the skin of the whole of the back was given 24 applications of approximately 0·25 ml. of a 0·075% solution of croton oil in acetone during a period of 26 weeks, at weekly intervals except for

TABLE 2.

Yields of tumours in mice injected with carbamates and followed by promoting treatment with croton oil.

Experiment	Carbamate injection	Dose mg.	Survivors after injection	Number of mice	Surviving mice: Mice with tumours: Total	Left side	Mid-line	Right side	Number of tumours: Total	Left side	Mid-line	Right side
I	Methyl	40	40	25	1	0	0	1	2	0	0	2
	Ethyl	25	40	30	16	9	2	13	55	13	3	39
	n-Propyl	20	39	26	5	2	0	3	5	2	0	3
	isopropyl	20	39	32	4	2	0	3	5	3	0	4
	n-Butyl	10	36	15	3	2	1	2	5	2	1	2
	Ethyl *N*-methyl	20	40	33	13	5	2	10	42	8	2	15
	Ethyl *N*-ethyl	50	32	29	6	2	2	4	12	3	2	7
	Ethyl *N*-n-propyl	12·5	30	30	1	1	0	3	4	1	0	3
III	Ethyl *N*,*N*-dimethyl	20	32	26	1	0	1	0	1	0	1	0
	Ethyl *N*,*N*-diethyl	15	30	21	2	1	1	0	3	1	2	0
	*Ethyl *N*,*N*-dimethyl	25	38	28	0	0	0	0	0	0	0	0
	*Ethyl *N*,*N*-diethyl	19	36	22	2	1	0	2	3	1	0	2
I & III	Controls		80	41	2	1	1	1	3	1	1	1

* Dose given in peanut oil by stomach tube.
40 mice in each group dosed with carbamates.

a 2 weeks' break after the 16th application. The number of mice with tumours and the distribution of the tumours were recorded 1 week after the last application of croton oil. The experiment was terminated by a laboratory accident which randomised the mice.

Experiment II. This experiment replicated experiment I except that the promoting treatment, that is the repeated applications of croton oil after the injection of the carbamates, was omitted. The animals were not randomly distributed between experiments I and II. The experiment was terminated after 26 weeks.

Experiment III. The experiment I was repeated using five groups of 40 mice, one of which was injected with 20·0 mg. ethyl *N,N*-dimethylcarbamate, the second with 15·0 mg. ethyl *N,N*-diethylcarbamate, the third was given 25·0 mg. ethyl *N,N*-dimethylcarbamate in 0·3 ml. peanut oil by stomach tube, the fourth 19·0 mg. ethyl *N,N*-diethylcarbamate in 0·3 ml. peanut oil by stomach tube and the fifth used as a control with croton oil promoting treatment only. The experiment was terminated after 26 weeks.

RESULTS.

Toxicity of carbamates.

The results of the toxicity tests are set out in Table I. Over the range of carbamates used the LD_{50} at 24 hr. decreased, and the rapidity of onset of acute toxic deaths, as evidenced by the approach of the LD_{50} at 2 hr. to the LD_{50} at 6 hr. and at 24 hr., increased firstly with each increase in chain length of the ester group, secondly, with substitution of an amide hydrogen of the ethyl ester by methyl, ethyl or n-propyl groups and with increasing chain length of the substituting group, and thirdly, with substitution of both hydrogen atoms in the *N*-position by methyl or ethyl groups. No significant difference was found between the n-propyl and isopropyl esters, although the former was more rapid

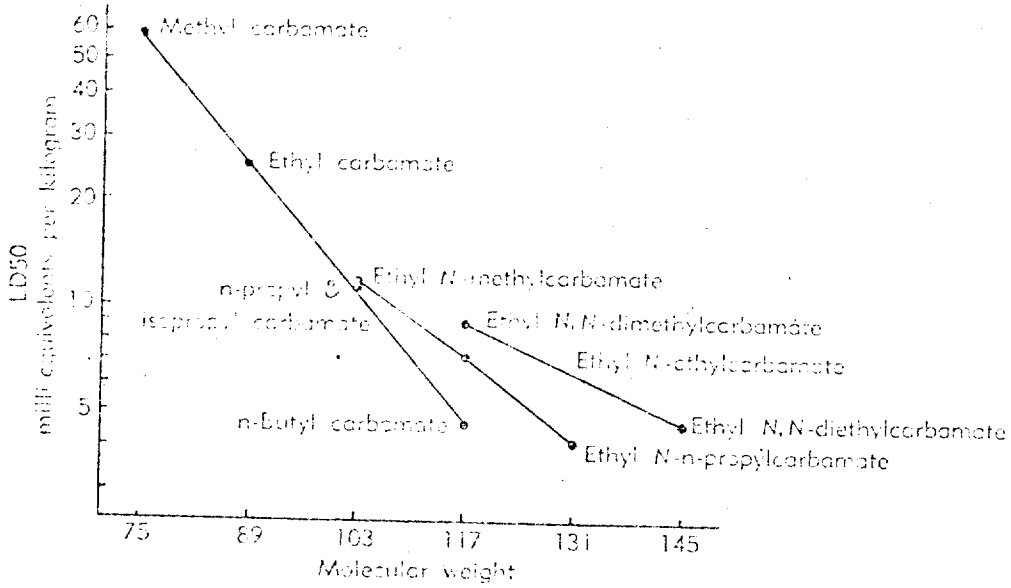

Fig. 1. Variation of LD_{50} of carbamates plotted logarithmically, with molecular weight.

in its effect. These characteristics appear to vary regularly with the molecular weight of the compounds but only to a lesser degree with structure, as can be seen from the slightly different positions and slopes of the LD_{50}-molecular weight curves of the homologous ester series and the *N*-substituted series, plotted in Fig. 1.

The narcotic properties vary with the same factors. Methyl carbamate is a relatively inefficient narcotic even at the LD_{50}. The higher homologues are efficient narcotics, the narcotic dose in the case of ethyl carbamate is about half the LD_{50} and as the molecular weight of the carbamate increases the narcotic dose approaches the LD_{50}. The ethyl *N*-substituted compounds fall into the same general pattern. On the other hand, the disubstituted ethyl esters are inefficient narcotics even at the LD_{50}. They produce a prolonged toxic lethargic state which, while graded as narcosis in Table 1, is clinically different and which is found only after dosages that lead to death of some animals in the group.

Examination of Table 1 shows that the state of narcosis persists longer with the methyl and propyl carbamates than with the ethyl ester, and is perhaps longer with the isopropyl than the n-propyl isomer. Butyl carbamate leads to a narcosis of very short duration. Substitution of a methyl group for one of the hydrogen atoms in the *N*-position of ethyl carbamate perhaps increases the duration of the narcosis but substitution of ethyl or n-propyl groups appears to reduce it. For reasons noted above, the behaviour of the *N*-disubstituted ethyl carbamates is ambiguous in this respect. While the assessment of the duration of narcosis is highly subjective, the differences are sufficiently clear to suggest that butyl carbamate is metabolised or excreted much more rapidly than ethyl carbamate, that methyl and propyl carbamates are metabolised more slowly and that the other *N*-substituted compounds appear to be metabolised at an approx. similar or more rapid rate.

Tumour initiating properties of the homologues of ethyl carbamate.

The yields of tumours in the animals injected with the several carbamates when followed by promoting treatment with croton oil, Experiments I and III, are shown in Table 2. This shows the total number of surviving mice with tumours and the total number of tumours. The results are dissected to show the number of mice with tumours and the number of tumours on the left side of the back, in the mid-line and on the right side that had the preliminary treatment with acetic acid 18 hr. before injection of the carbamates, for the reasons described previously (Pound and Withers, 1963). The control groups treated with croton oil only in the two experiments are combined in the Table.

In the groups of animals injected with the carbamates alone without promoting treatment with croton oil, Experiment II, only one mouse developed a papilloma during the 14th week from the group injected with ethyl carbamate on the side that had previously been treated with acetic acid. It is clear, therefore, that

none of the compounds produced any significant yield of tumours when acting alone and the results therefore are not tabulated.

On comparing the total yields of tumours in the various groups with that in the control groups, Table 2, the results show significant yields of tumours with ethyl carbamate ($X^2 = 65$, 1 d.f., $P < \cdot 001$), ethyl *N*-methylcarbamate ($X^2 = 22 \cdot 5$, 1 d.f., $P < \cdot 001$), ethyl *N*-ethylcarbamate ($X^2 = 9 \cdot 2$, 1 d.f., $P < \cdot 005$) and butyl carbamate ($X^2 = 5 \cdot 2$, 1 d.f., $P < \cdot 025$). The yields with n-propyl and isopropyl carbamates are not significant at the 5% level ($X^2 = 1 \cdot 9$, 1 d.f., $\cdot 25 > P > \cdot 1$; and $X^2 = 2 \cdot 8$ 1 d.f., $\cdot 1 > P > \cdot 05$, respectively) and the yields with ethyl *N*-n-propylcarbamate, ethyl *N,N*-dimethylcarbamate and ethyl *N,N*-diethylcarbamate and methyl carbamate are not significant. The yield produced by ethyl carbamate is significantly greater than that produced by the ethyl *N*-methylcarbamate ($X^2 = 14 \cdot 1$; 1 d.f., $P < \cdot 001$); and the tumour yield from ethyl *N*-methylcarbamate is greater than that from ethyl *N*-ethylcarbamate but the difference is of doubtful significance ($X^2 = 3 \cdot 1$, 1 d.f., $\cdot 1 > P > \cdot 05$).

The number of tumours on the right side of the skin of the back which had the preliminary treatment with acetic acid is greater than on the left untreated side in the mice given ethyl carbamate ($X^2 = 13$, 1 d.f., $P < \cdot 001$), as previously reported (Pound, 1966). In the case of ethyl *N*-methylcarbamate and *N*-ethylcarbamate, which both gave significant total yields of tumours, the yield was greater on the side that had the preliminary treatment but not significantly so ($X^2 = 2 \cdot 1$, 1 d.f., $P > \cdot 1$; and $X^2 = 3 \cdot 6$, 1 d.f., $\cdot 1 > P > \cdot 05$, respectively). In the case of the other carbamates, the yield on the two sides did not differ.

DISCUSSION.

The LD_{50} values of the carbamates recorded in this paper are higher than those reported by Skipper, Bryan, Riser, Welty and Stelzenmuller (1948) for the same compounds given intraperitoneally in CFW strain mice, particularly for methyl carbamate. The exponential variation of the LD_{50} with molecular weight, Fig. 1, is similar to the variation of narcotic and toxic effects for various organisms and bacteria of a variety of series of homologous compounds of differing chemical nature. Narcotic properties in general have been regarded as non-specific actions of drugs due to accumulation at cell membranes rather than to specific chemical actions on particular enzymes in the cell (Butler, 1950); they have been related, in thermodynamic terms, to the free energy of absorption at cell membranes (Ferguson, 1939; Brink and Posternak, 1948). This does not preclude an indirect interference with metabolic systems within the cell; indeed the acute toxic properties appear to result from such disorganization of the chain of respiratory enzymes.

None of the carbamates produced tumours when administered alone, but some of them did so after promoting treatment with croton oil. In terms of the two stage hypothesis (Salaman, 1958), the active members therefore act, like

urethane, as initiating agents. Ranked in order of the proportion of tumours produced in the surviving mice, the apparent order of potency was ethyl carbamate, ethyl *N*-methylcarbamate, ethyl *N*-ethylcarbamate, butyl carbamate, n-propyl and isopropyl carbamate with doubtful effect, and ethyl *N*-n-propylcarbamate, ethyl *N,N*-dimethylcarbamate, ethyl *N,N*-diethylcarbamate and methyl carbamate without significant effect. A similar grading of initiating effect was reported (Berenblum *et al.*, 1959) in the case of ethyl carbamate, ethyl *N*-methylcarbamate, propyl carbamate (n-propyl or isopropyl ester not stated), ethyl *N,N*-dimethylcarbamate and methyl carbamate, when injected into mice in doses not greatly dissimilar to those in the present work.

These apparent relative potencies may be affected by the dosage of each compound, as the action of urethane varies with dose (Boe and Salaman, 1954; Berenblum and Haran-Ghera, 1957). Thus an increased dose of the higher homologues or *N*-substituted derivatives of ethyl carbamate, if this was not limited by their toxicity, would increase their effect. To take the other point of view, the reported variation of tumour yield with dosage of urethane (Berenblum and Haran-Ghera, 1957) suggests that reduction of the dose of urethane to one-third of that used (that is to a dose approx. equivalent to that of the ethyl *N*-n-propylcarbamate and *N,N*-diethylcarbamate, would still produce a significant yield of tumours. This factor, therefore, is unlikely to upset the general order of potencies. On the other hand, it may be affected by the rate of elimination of the compounds, since, if proliferating cells are more susceptible to the initiating activity of carbamates (Pound, 1966), the number affected would be conditioned by the number proliferating during the period in which the carbamate, or an active metabolite, was present in an effective concentration. The rate of metabolism of methyl carbamate (Boyland and Papadopoulos, 1952) is slower than that of the ethyl ester (Boyland and Rhoden, 1949), as suggested by the relative durations of their toxic effects. The metabolic rates of the other compounds, therefore, also probably vary in the manner suggested by the duration of narcosis. If slower than ethyl carbamate, this factor would tend to increase the apparent relative effects. In the case of butyl carbamate, which appears to be metabolized very rapidly, the apparent initiating effect might be significantly reduced. Clearly these considerations require further data than is available at present for a final appraisement but, tentatively, the above order may be considered a reasonable approximation to the real order of activity.

The skin tumour-initiating property of carbamates, therefore, does not vary regularly with their molecular weight nor with their narcotic potencies, and is not likely to be related to the physico-chemical actions on cells which result in their narcotic and acute toxic properties. This view is supported by the fact that administration of lysergic acid diethylamide to mice with urethane, thereby obviating the narcotic effect, did not alter the tumour yields (Berenblum, Blum and Trainin, 1959).

On the contrary, definite structural features in the carbamate molecule appear to be concerned. Firstly, the tumour-initiating property appears to be conditioned by the nature of the ester group. Ethyl carbamate is most active and the butyl ester also appears to have significant activity, whereas, of the odd chained esters, methyl carbamate is without effect and the propyl esters have a doubtful effect. Secondly, it appears likely that it is necessary for at least one free hydrogen to be present in the amide group since ethyl *N*-methylcarbamate and ethyl *N*-ethylcarbamate were effective, while the disubstituted compounds were not. Isopropyl *N*-phenylcarbamate and isopropyl *N*-chlorophenylcarbamate which have been reported to have initiating properties (Van Esch, Van Genderen and Vink, 1958) also have an unsubstituted hydrogen in this position. Thirdly, the nature of a substituent on the nitrogen atom influences the tumour yield.

It is of interest that β-hydroxyethyl and β-aminoethyl carbamates, as well as the *N*-carboethoxy derivatives of glutamic acid, aspartic acid, alanine and glycine which may be regarded as *N*-substituted ethyl carbamates, were shown to be inactive while ethyl *N*-hydroxycarbamate was a potent initiator (Berenblum *et al.*, 1959). *N*-hydroxy derivatives of methyl, ethyl, n-propyl and n-butyl carbamates have been identified as metabolites of these compounds in rats and rabbits (Boyland and Nery, 1965) and it has been suggested that the metabolite *N*-hydroxyurethane rather than urethane itself might be the actual initiating agent in mice. The necessity for an unsubstituted amide hydrogen for initiating activity and the influence of the substituting group might be significant in this regard. However, *N*-hydroxyurethane has not been proved to be a metabolite of urethane in mice (Mirvish, 1964, 1966).

The effect of prior stimulation of the skin with acetic acid in increasing the yield of tumours initiated by urethane was expected from previous results (Pound, 1966). Prior stimulation with acetic acid produced an increased yield of tumours with ethyl *N*-methylcarbamate and ethyl *N*-ethylcarbamate, although the increase was of doubtful significance; apart from ethyl carbamate these were the most active initiators. No general deductions can be made from these results which, however, point to the need for further investigation. It was envisaged that, with prior stimulation by acetic acid, tumour-initiating activity which otherwise would escape detection might be disclosed with some of the less active carbamates.

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Browning of Starch from Indian Tubers

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0.1% Acetic, citric, tartaric and oxalic acids have been found to prevent the browning of starchy tubers. Treatment with zinc and HCl is the most efficient process for this purpose. Low *p*H is necessary but it by itself cannot prevent browning. A mechanism for the prevention of browning is suggested and discussed.

It had been reported earlier[1] that 10% acetic acid prevents the browning of sweet potato starch. It may now be confirmed that the starch treated as above has been kept for two years under the ordinary conditions of temp., pressure and humidity without showing any signs of browning or deterioration. It was considered desirable to investigate it further in order to find (i) the lowest concentration of acid necessary to prevent browning, (ii) whether this process could be used for other starches, (iii) what other substances could prevent the browning, (iv) the role of *p*H, (v) the role of halides and (vi) the mechanism of the reaction. Though a definite answer can not be given to all these questions yet it was considered desirable to publish the results obtained.

(i) *Minimum concentration necessary* :—Experiment No. 2 shows that 0.1% acetic acid is sufficient to prevent browning.

(ii) *Effect on other starches* :—The other starchy tubers tried were the common tubers of India i.e. potato (*Solanum tuberosum*), Arvi (*Colocasia antiquorum*), Zaini kand (*Dioscorea bulbifera*) and Ratalu (*Dioscorea sativa*). As experiment III shows, except for Arvi, 0.1% acetic acid is efficient for all other starches.

(iii) *Other substances for prevention of browning* :—A large number of substances was studied for this purpose. Except for organic acids no other substance was found efficient for this purpose (Expt. 1).

(iv) *The role of pH* :—Low *p*H appears to be necessary but not sufficient for the prevention of browning. Dilute hydrochloric, sulfuric and nitric acids failed to have any beneficial influence despite their low *p*H. (Expt. 1).

(v) *Comparative efficiency of different organic acids* :—Acetic, tartaric and citric acids have almost identical effect while oxalic acid appears to have a much greater effect. (Expt. 4).

(vi) *Effect of halides* :—Owing to a prevailing idea that halides exert a beneficial influence on the prevention of browning, their effect was re-investigated with great care. It was found that neither alone nor in conjunction with 0.05% acetic acid did they exert any beneficial influence (Expt. 1 and 2).

Successful prevention of browning appears to depend on 4 factors :—(i) low *p*H, (ii) complexation, (iii) reduction and (iv) permeability.

Since no successful prevention of browning is obtained at high *p*H and since in all the successful methods for the prevention of browning *p*H has to be lowered it follows that a low *p*H is necessary for this purpose. The low *p*H must be complemented either with reducing action or with complex formation in order to prevent browning. This inescapable conclusion follows from the study of all the relevant data : and the action of all the agents efficient in the prevention of browning can be explained on this basis.

Thus hydrochloric, nitric and sulfuric acids can not prevent browning because they satisfy only one condition i.e. low *p*H. They are neither reducing nor complexing agents. On the other hand, the complex forming ability of acetic, citric, tartaric and oxalic acid is well known. Hence the organic acids function as efficient preventing agents for browning. Oxalic acid is at the same time a reducing agent also. Hence oxalic acid behaves more efficiently than the other organic acids. Sulfur dioxide is also known to prevent browning. Its action is to be expected owing to the fact that here we have low *p*H together with reducing properties. A new experiment was tried to test this hypothesis. As has been said above, hydrochloric acid does not prevent browning. However when freshly cut potato and sweet potato chips were treated for fifteen minutes with Zn and HCl, browning was completely prevented. This strongly lends support to the fact that low *p*H together with reducing action is necessary for the successful prevention of browning and provides a very simple criterion to predict the efficacy of a reagent. Also any reagent which has got complex forming ability must be useful if used at a low *p*H. Ethylene diamine tetraacetic acid should be one of these. It will be interesting to try this reagent to see its efficacy.

Permeability appers to be another important factor in this respect. When potato chips are boiled with alcohol their surface remains white but from inside they turn brown. The reason is that alcohol has low permeability and hence on boiling it affects only the outer surface.

It has been shown by Clayson[2] that the surfaces treated by dilute acetic acid lose water in preference to acetic acid and thus the acid becomes more concentrated. In our opinion the acetic acid solution reacts with the basic substances already present in natural starch to form loose complexes. The reaction may be represented by the simple equation : $xB + yCH_3COOH = [CH_3COOH]_y\,[B]_x$. Owing to the high concentration of acetic acid the reaction is displaced to the right. However, on drying acetic acid must exist in a loose combination with B. The complex forming ability of acetic acid changes the nature of the starch surface which is now no longer susceptible to attack by browning enzymes. Even in the rainy reason when atmospheric moisture increases, there is no browning as the complex hydrolyzes giving acetic acid back which remains on the surface and exerts a protective influence on the starch granules.

It has been shown by Gormputra and Fabian[3] that 1% acetic acid is more effective than 1% mono, di, or trichloroacetic acid. This is obvious on the basis of what has been said above. The replacement of hydrogen atoms of acetic acid results in a great change in the electron distribution of the acid

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molecule and this electron distribution greatly affects the properties of the carbonyl group and reduces its complexing power.

It has been reported[4] that polyphenol peroxidase is mainly responsible for the browning of apples and that chlorogenic acid acts as an active substrate for polyphenol peroxidase in sweet[5] potato and white[6] potato. If these observations are correct then the agents which are successful in preventing the browning of the sweet potato and potato starch should also effectively prevent the browning of apples. However repeated efforts failed to produce any results. Even 10% acetic acid which completely arrests the browning of starchy tubers failed to have any significant effect on the browning of apples. It therefore appears that the active agent responsible for the browning of starchy tubers is different from the agent which causes the browning of apples.

EXPERIMENTAL

The sweet potato washed and cut into thin slices was treated with the reagents mentioned below for 24 hrs. After this period, the slices were removed from the solution and dried in shade with results tabulated below. A negative sign means that browning took place on the slices while a positive sign means no browning.

Experiment No. 1

Reagent (Conc. 10%)	Result	Reagent (Conc. 10%)	Result
Sodium hydroxide	–	Sodium chloride	–
Hydrochloric acid	–	Sodium bromide	–
Nitric acid	–	Sodium iodide	–
Sulfuric acid	–	Ammonium chloride	–
Glycerine	–	Tartaric acid	+
Hydrogen peroxide	–	Citric acid	+
Borax	–	Oxalic acid	+

Experiment No. 2

Reagent	Conc.	Result
Acetic acid	10%	+
" "	5	+
" "	3	+
" "	1	+
" "	0.5	+
" "	0.1	+
" "	0.05	–

Experiment No. 3

Reagent	Starchy tuber	Colour on drying
0.1% Acetic acid	Sweet potato	No colour
0.1% Tartaric acid	" "	"
0.1% Citric acid	" "	"
0.1% Oxalic acid	" "	Higher than original
0.1% Acetic acid	Potato	No colour
0.1% Tartaric acid	"	"
0.1% Citric acid	"	"
0.1% Oxalic acid	" "	Higher than original
0.1% Acetic acid	Arvi	Very slight colour
0.1% Tartaric acid	"	"
0.1% Citric acid	"	"
0.1% Oxalic acid	"	"

The results with Rataln and Zami Kand were identical with potatoes and sweet potatoes.

Experiment No. 4

Reagent	Concentration	Colour of apples	Remarks
Acetic acid	0.1%	Dark brown	No effect
"	0.5%	"	"
"	1.0%	"	"
"	5.0%	Brown	Very slight effect
"	10.0%	"	"

Experiment No. 5

Freshly cut pieces of sweet potatoes	Treatment with Zn and HCl	Colour on washing with water	Colour on drying
Potatoes	15 min.	White	White
Arvi	"	"	"
Rataln	"	"	"
Zami Kand	"	"	"

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INCORPORATION OF [1-^{14}C]ACETATE INTO THE LIPIDS OF AORTAS OF DIFFERENT SPECIES

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SUMMARY

Total lipids and *in vitro* incorporation of [1-^{14}C]acetate into different lipid fractions of abdominal and thoracic aortas of monkeys, rabbit, rat and chick have been compared. Percentage of total lipids in thoracic aorta was generally higher than in the abdominal part. Some differences were also observed in the incorporation of [^{14}C]acetate into lipids of the two segments of the aorta. Percentage of total lipids in monkey aorta was lower than in other species. Highest incorporation of radioactivity was observed in the phospholipid fraction, while that into the cholesterol fraction was poor in all the species studied. Incorporation of radioactivity into all lipid fractions of aorta was considerably higher in chick than in other species. Incorporation into the cholesterol fraction occurred consistently only in chick aorta while in other species incorporation was absent or negligible. Monkey aorta incorporated [^{14}C]acetate relatively more into the phospholipid fraction than did the aortas of other species. Radioactivity in total lipids, cholesterol, free fatty acid and the triglyceride fraction was lower in monkey aorta than in rat or rabbit aorta. The significance of these findings in relation to the susceptibility of these species to atherosclerosis is discussed.

INTRODUCTION

Though there have been many isolated studies devoted to the metabolism of cholesterol, free fatty acids or phospholipids in normal aorta (for literature see ref.[1]), there are few studies in any one species that have been designed to obtain an integrated picture of synthesis of all the lipids from acetate precursor[2-4]. Each group of workers studied only one species and, considering the experimental variables, it is difficult to compare the results obtained with these various species. Therefore, in this

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study, the pattern of *in vitro* incorporation of [1-^{14}C]acetate into the different lipids by the aorta was investigated in monkeys, rabbits, rats and chicks.

MATERIALS AND METHODS

Animals

The experimental animals were normal adult males on nutritionally adequate stock diets:

(1) Six Rhesus monkeys (*Macaca radiata*) weighing between 3.7 and 6.4 kg;

(2) Five groups of six albino rats each weighing 135–424 g;

(3) Seven rabbits weighing 1.6–2.0 kg; and

(4) Six white Leghorn cocks with weights ranging from 1.8–2.3 kg.

Reagents

Using analytical grade chemicals, Krebs-Ringer-bicarbonate buffer was prepared so that 3 ml of this when mixed with 1 ml of 0.9 % NaCl had the prescribed composition[6]. In addition the incubation medium contained glucose (2.7 mg/ml).

[1-^{14}C]-Sodium acetate (specific activity: 1.2 mC/mole) was obtained from the Bhabha Atomic Research Centre, Bombay, India. This was dissolved in sterile 0.9 % NaCl solution to yield a solution containing 3 μC/ml.

The organic solvents used in these studies were of analytical grade or purified by distillation. Silica gel G (Merck) was used for thin layer chromatography (TLC).

Dissection

The animals were fasted overnight before sacrifice. Rats were anaesthetized by inhalation of diethyl ether and monkeys by an intravenous injection of sodium 5-ethyl-5-(1-methylbutyl)-2-thiobarbiturate (Intraval Sodium) at a dose of 30 mg/kg body weight. Rabbits and chicks were bled to death by cutting the blood vessels in the neck. Either under deep surgical anaesthesia or immediately after death, the aorta was dissected out and cut into two parts—the thoracic from the left ventricle to the diaphragm and the abdominal from the diaphragm to the internal iliac arteries. Both parts were then transferred to ice-cold water. There, the artery was dissected from its branches close to their origin. The adhering tissues were removed from the aorta. As the monkey aorta was large, each segment (thoracic or abdominal) was divided longitudinally into two halves and incubated separately, but duplicates were pooled after incubation and treated as a single sample thereafter. As soon as each piece of aorta was ready, it was transferred to ice-cold normal saline and kept there until incubation. The average time lag between killing the animal and incubating the aorta was 45 min for monkeys, 24 min for rabbits, 25 min for chicks and 115 min for each group of six rats. A pool of six aortas was incubated in each flask in the case of rats. Only 3 aortas were used in the case of other animals. Thoracic and abdominal

Incubation

The aortas were transferred immediately to 50 ml conical flasks containing 3.0 ml Krebs-Ringer-bicarbonate buffer and 1 ml of radioactive acetate solution. The flasks were incubated at 37.5 °C under an atmosphere of 95 % O_2–5 % CO_2 for 3 h; the flasks were shaken in the meanwhile.

After incubation the tissues were removed from the flask, washed thoroughly with 10 % sodium acetate solution to remove labelled acetate from the surface and dried between filter papers. Their weights were quickly recorded and they were then dropped into 5 ml of methanol–chloroform (1 : 2, v/v) to extract the lipids. The two segments from monkey aorta were pooled at this stage. After keeping overnight, the solvent was decanted and the tissues were refluxed with a fresh lot of methanol–chloroform mixture for 3 h over a water bath. This procedure ensured complete extraction of the lipids. The combined lipid extract was concentrated and washed according to FOLCH *et al.*'s[6] procedure for removing water-soluble radioactive compounds such as acetate. The purified extract was evaporated in a conical flask, dried in a desiccator and weighed to obtain the total lipid content of aortas.

The lipids were dissolved in 2.5 ml of methanol–chloroform mixture and 0.5 ml of this solution was used for determining total lipid radioactivity. Two ml of the extract were concentrated to a small volume and applied quantitatively as a streak on a TLC plate of Silica Gel G (300–500 μ thick); the lipid classes were separated using the solvent system, petroleum ether–ethyl ether–acetic acid (170 : 40 : 4, v/v/v). Lipid bands were detected with 2,7-dichloro-fluorescene, scraped out separately and the lipids were extracted in a flow column. Neutral lipids were extracted with 25 ml of chloroform, while phospholipids were recovered with the series of solvents suggested by SKIPSKI *et al.*[7]. The procedure for extraction was standardized for maximal elution of radioactivity as indicated by the absence of radioactivity in the eluent on further extraction. Total radioactivity recovered, however, only ranged from 50–70 %. An investigation into the incomplete recovery suggested that at least a part of the lipids was bound to silica gel and was not extractable by the usual lipid solvents. Poor recovery of lipids from silica gel has also been reported by other workers[8].

The extracts from the silica gel were concentrated and transferred to cupped planchets and the solvents evaporated off. Radioactivity of samples was counted in a windowless gas-flow G. M. Counter.

RESULTS

Weight of aorta, its dry weight and lipid content are given in Table 1. Radioactivity in total lipids and various lipid fractions of aorta, expressed on the basis of dry matter or lipid content, is shown in Table 2 and Fig. 1. The figure also includes the percentage distribution of radioactivity in different lipid fractions. Despite incomplete recovery, there were no differences in the recovery of radioactivity from aortas of different species; hence their radioactivities were comparable.

Species	Body weight (average)	Aorta					
		wet weight (mg)	Total protein (as albumin) (mg)	Total lipids wet weight	Total lipids (dry matter)	Total radioactivity (cpm/g dry matter)	Total radioactivity (cpm/g total lipids)
Monkey	5.2 kg	T[b] 777.6	201.0	32.3 ± 0.53	15.6	5.2 ± 0.36	
	-do-	A[b] 265.3	74.1	29.2 ± 0.77	4.9	6.7 ± 0.81	
Rabbit	1.9 kg	T 134.7	47.3	30.6 ± 0.83	8.2	10.7 ± 1.83	
	-do-	A 65.7	18.7	29.2 ± 1.12	4.8	20.2 ± 6.97	
Rat (5 groups)	233 g	T 91.0	36.0	40.4 ± 2.68	4.4	10.0 ± 2.95	
	-do-	A 92.9	23.0	32.7 ± 1.09	6.0	23.0 ± 7.11	
Chick	2.0 kg	T 250.8	72.2	27.8 ± 0.75	11.6	16.3 ± 2.15	
	-do-	A 128.0	36.0	28.1 ± 0.93	12.7	37.2 ± 6.84	

Means ± S.E.M.
T: thoracic; A: abdominal.

cept in one group of rats. Percentage of dry matter in the thoracic segment was significantly higher than in the abdominal segment in the monkey ($P < 0.01$) and rat ($P < 0.05$) but not in rabbit or chick.

The percentage of total lipids in thoracic aorta was generally lower than that in the abdominal segment. This difference was significant ($P < 0.001$) in monkey, chick and rat, but not in rabbit. A comparison of total lipid radioactivity (per mg of dry matter) in the two segments indicated that monkeys had a significantly higher ($P < 0.05$) radioactivity in the thoracic segment while rabbit showed a significantly higher ($P < 0.05$) value in the abdominal segment; no significant differences were observed between the two segments in rat and chick. The distribution of radioactivity in different lipid fractions in thoracic and abdominal aortas did not differ significantly in

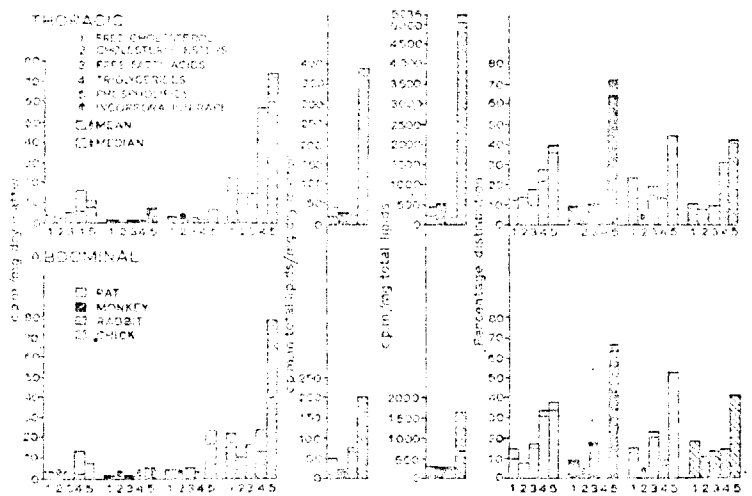

Fig. 1. Incorporation of [14C]acetate into different lipids in various species.

any of the species studied, except for triglyceride fractions in the chick. Total incorporation and percent distribution of radioactivity in the triglyceride fraction were significantly higher in chick thoracic aorta than in its abdominal part.

A comparison of the four species indicated that the percentage of total lipids in the two segments of aorta was significantly lower ($P < 0.05$, $P < 0.001$) in monkey than that in rabbit, rat or chick; there were no differences among the last three species. The highest incorporation of radioactivity was observed in chick aorta. The difference between the value observed in chick and other species was highly significant ($P < 0.001$) in the case of the thoracic segment only. Among the other species, incorporation of radioactivity (cpm/mg dry matter) into the thoracic aorta was significantly higher ($P < 0.001$) in monkey than in rabbit.

Incorporation of [14C]acetate into all lipid fractions of the chick aorta was considerably higher than in other species. These differences were highly significant ($P <$

[illegible] IN THE [illegible] ACROSS IN THE [illegible] SPECIES

[illegible]	[illegible]	Cpm/mg D.M.											
		Total lipids		C	CE	FFA-1		TG		PL			
T	565 ± [illegible]	30 ± 3.91	1.2 ± 0.31	0.4 ± 0.07	1.0 ± 0.09	1.0 ± 0.29	84 ± 2.29
A	268 ± 40.5	19 ± 3.1	0.6 ± 0.10	1.59	1.1 ± 0.19	0.6 ± 0.21	1.9 ± 1.21
T	176 ± 11.8	25.2 ± 4.30	3.4 ± 0.70	3.1b	2.8 ± 0.96	2.3 ± 0.95	7.1 ± 1.97
A	821 ± 115.8	67.1 ± 16.22	3.6 ± 1.04	24.4b	4.9 ± 1.46	3.2 ± 0.48	25.1 ± 10.50
T	425 ± 259.5	42.5 ± 27.8	4.1 ± 4.20	1.0b	4.3 ± 1.15	15.8 ± 11.70	10.8 ± 5.19
A	296 ± 149.9	49.7 ± 38.16	3.0 ± 1.09	1.4b	3.2 ± 1.48	13.2 ± 11.72	0.8 ± 2.68
T	2813 ± 477.2	383 ± 53.0	22.5 ± 3.79	11.8 ± 1.48	15.3 ± 1.35	33.0 ± 8.84	74.7 ± 11.37
A	823 ± 436.0	207 ± 72.5	22.1 ± 7.39	10.2 ± 2.92	15.7 ± 5.15	23.8 ± 10.98	78.7 ± 38.13

[illegible] ± S.E.M.

[illegible] observed only in a few cases.

[illegible]: cpm: counts per minute; D.M.: fat-free dry matter; T: thoracic; A: abdominal; C: cholesterol; CE: cholesterol esters; FFA: free fatty [illegible]; TG: triglycerides; PL: phospholipids.

rat. Chick aorta incorporated significantly more [^{14}C] into triglycerides ($P < 0.05$) and phospholipids ($P < 0.01$) than did rat aorta. Measurable incorporation of radioactivity into cholesterol and cholesterol esters occurred consistently only in chick aorta, while incorporation in other species was absent or negligible. Least incorporation of radioactivity into free cholesterol and free fatty acids or triglycerides occurred in monkey aorta compared with rat or rabbit. There were no significant differences in the incorporation into various lipid fractions between rat and rabbit aortas.

When radioactivity in different fractions was expressed as percentage of total activity, highest percentage activity was found in the phospholipid fraction and least in the cholesterol fraction in all the species studied. Percentage distribution of radioactivity in the cholesterol fraction (of thoracic lipids) was higher ($P < 0.05$–$P < 0.01$) in rabbit compared with other species, and that into free fatty acids was higher (though not significantly) in rat and rabbit compared with monkey and chick. Percent incorporation into phospholipids was higher ($P < 0.05$) in the case of the monkey than in the other species studied.

DISCUSSION

The present study attempts to compare the relative efficiency of the thoracic and abdominal parts of aorta from monkey, rabbit, chick and rat in their ability to incorporate [^{14}C]acetate into lipid classes *in vitro* under comparable conditions. It was observed that the recovery of radioactive lipids from TLC-plates, though similar in experiments with different species, was incomplete. This limitation has to be borne in mind while interpreting the results. Incorporation of [^{14}C]acetate was found to be highest in the phospholipid fraction and poorest in the cholesterol and cholesterol ester fractions. Higher incorporation of [^{14}C] from acetate into fatty acids and phospholipids[2,3] and low incorporation into cholesterol[4,10] has been observed earlier. Of the species studied here, much higher incorporation into all the fractions including cholesterol was observed in chick aorta.

AZARNOFF[11] found considerable incorporation of [^{14}C]acetate into aortic cholesterol of rabbit and chick, but not into that of rat particularly after purifying cholesterol by the debromination procedure. However, LOOMEIJER AND VAN DER VEEN[2] found some cholesterol synthesis in rat aorta, when the lipids were separated on silicic acid columns. Using similar techniques, some cholesterol synthesis was also evident in rat aorta in the present experiments. Unlike earlier work[11,12], distinct differences in aortic cholesterol synthesis were found here between rabbit and chick. In all species, other than rabbit, thoracic aorta showed higher incorporation of [^{14}C]acetate into total lipids but contained less lipids than abdominal aorta. In the present experiments, total lipid synthesis in rabbit aorta was found to be greater in the abdominal part than in the thoracic part. However, WHEREAT[4] did not find significant differences in [^{14}C]-acetate incorporation into total fatty acids between the thoracic and abdominal segments of rabbit aorta. DEAVY[13] observed greater incorporation of ^{32}P into phospholi-

acetate incorporation into phospholipids was not significantly different between the two species in any of the species studied in the present series. Observed differences in acetate incorporation may be taken to represent differences in the capacity of aorta to synthesize lipids. However, other factors, such as differences in aortic thickness and in size of aortic pool, might have partly contributed to the observed differences in the incorporation pattern, but their significance may be negligible.

It is recognized that local synthesis of lipids does not significantly contribute to lipid accumulation in the aorta. It is especially so perhaps with regard to cholesterol, where synthesis in aorta is negligible. However, phospholipid synthesis in aorta may be important in cholesterol solubilization and its removal from the arterial wall into plasma[11]. When comparing the pattern of ^{14}C acetate incorporation into aorta in the three species studied, local synthesis of lipid appears to be more prominent in chick than in aorta of other species. Monkeys showed proportionally higher incorporation into phospholipids than in other species. The significance of the varying *in vitro* incorporation in these species in terms of their susceptibility to atherosclerosis needs to be evaluated. It may be significant that in chicks which are susceptible to spontaneous atherosclerosis, unlike the other species studied, the level of lipid synthesis is quite high. The relatively higher rate of synthesis of phospholipids in monkeys, than in other species, may offer protection against aortic lipid accumulation.

The finding that ^{14}C acetate incorporation into total lipids and the triglyceride fraction of rat aorta is higher than that in rabbit aorta needs further explanation. This observation is not consistent with the rabbit's greater susceptibility to atherosclerosis. As lipoprotein lipase activity in rat aorta is greater than that in rabbit[15], this last enzyme may be a factor in aorta against lipid accumulation in spite of higher synthetic activity.

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716 J. Biol. Chem., 154(1): 311-312, 1944

THE UTILIZATION OF ACETIC ACID FOR FATTY ACID SYNTHESIS

Sir:

No information is available as to the intermediates in the biological synthesis of fatty acids from carbohydrates. In earlier experiments[1] with dueterio acetate the isotope concentrations found in the fatty acids were not significant. We have now prepared a sodium acetate containing 19.6 atom per cent excess C^{13} in the carboxyl group and a high concentration (77 atom per cent excess) deuterium in the methyl group. This acetate was fed to mice at a level of 1.6 mM per 100 gm. of body weight for 8 days. Fatty acids were then isolated from the pooled livers and skinned carcasses. A sample of respiratory CO_2 was obtained 15 minutes before the end of the experimental period. Glycogen (11.3 mg.) was isolated from the livers.

From the total fatty acids of the liver (238 mg.), 48 mg. of saturated fatty acids were isolated. After their analysis for C^{13} and D, the remaining 44 mg. were decarboxylated[2] in order to determine the concentration of C^{13} in the carboxyl group. The fatty acids of the skinned carcasses (2.3 gm.) were separated in a similar manner. The isotope concentration of the various fractions are given in the table.

Sodium Acetate Fed, 9.8 Atom Per cent Excess C^{13}, 77 Atom Per Cent Excess D

	Atom per cent excess			
	Carcass		Liver	
	C^{13}	D	C^{13}	D
Total fatty acids	0.081	0.15	0.166	0.32
Saturated fatty acids	0.101	0.24	0.160	0.42
Carboxyl carbon of saturated fatty acids	0.179		0.299	
Glycogen			0.038	

The dueterium concentration of the body fluids at the end of the experiment was 0.69 atom per cent excess. The respiratory CO_2 during the last 5 minutes contained 0.056 atom per cent excess C^{13}.

A similar experiment was carried out with growing rats. They were fed for 3 days acetate containing 9.8 atom per cent excess C^{13} at a level of 1.6 mM per 100 gm. of body weight. The total fatty acids contained 0.010 atom per cent excess C^{13} and the carboxyl carbon of the total acids contained 0.030.

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In connection with these findings, the work of Sonderhoff and Thomas with yeast[1] and the short time experiments of Buchanan, Hastings, and Nesbett[2] with rats are of interest. The results here reported furnish positive evidence for the utilization of the carbon of the carboxyl group of acetic acid for fatty acid synthesis.

On complete combustion, the carbon of the diet of the mice (0.5 atom-g. carbon per day per 100 gm. of body weight) should yield CO_2 with an average C^{13} concentration of about 0.07 atom per cent excess. As the concentrations found in the fatty acids are in all cases higher than this value, the results cannot be due to CO_2 assimilation. Moreover, as after the feeding of the deuterio acetate for 8 days the fat had a deuterium concentration of 1.5 to 1.7 times that of the body fluids, utilization of the methyl as well as the carboxyl groups of acetate must have occurred. The fact that the C^{13} concentration of the carboxyl carbon atom is approximately twice that of the total molecule suggests that the fatty acids are synthesized by successive condensations of C_2 units.

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J. Biol. Chem. 157: 749-750 (1945)

SOME BIOLOGICAL REACTIONS OF ACETIC ACID

Sirs:

The ability of the animal organism to synthesize acetyl groups and rapidly oxidize acetic acid raises the question as to the mechanism by which acetate is metabolized. It has been suggested that acetate or acetoacetate can enter the "citric acid" cycle by condensation with oxalacetate.[1] Citric acid formation from acetate and oxalacetate has been observed in yeast[2] and from acetoacetate and oxalacetate in kidney.[3] The intermediates of the "citric acid" cycle, ketoglutarate, oxalacetate, etc., cannot be isolated from the intact animal but glutamic and aspartic acids, which are in biological equilibrium with these keto acids, can be isolated from protein. We have fed labeled acetate containing 19.6 atom

C^{13} Concentrations in Liver Constituents in Atom Per Cent Excess

	Rats (fed labeled acetate for 3 days)	Mice (fed labeled acetate for 5 days)
Glutamic acid	0.035	0.080
α-Carboxyl of glutamic acid	0.041	0.096
Aspartic acid	0.023	0.038
Glycogen	0.020	0.038
Respiratory CO_2		0.066
Amidine carbon of arginine		0.080
Urinary urea	0.035	

per cent excess C^{13} in the carboxyl group to both rats and mice and have found the liver dicarboxylic amino acids to contain C^{13}. Degradation by ninhydrin or chloramine-T of the glutamic acid shows that not all of the C^{13} is contained in the α-carboxyl group. The data are shown in the table; similar, though lower, values were found in the carcasses. While some of the C^{13} could have been introduced by CO_2 fixation, this mechanism cannot account for the C^{13} distribution we find. The findings indicate that a pathway exists by which acetate is utilized to form intermediates of the carbohydrate metabolism. As the tissue proteins have a high content of glutamic and aspartic acids, and as large quantities of ketoglutaric acid and oxalacetic acid arise from protein and carbohydrate metabolism, high C^{13} concentrations cannot be expected in the dicarbox-

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ylic amino acids, even if all the acetate were utilized in the formation of these compounds.

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Effect of Physiologic Acids on Electrolytes in Rat Diaphragm[1]

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ABSTRACT

ROGERS, TERENCE A. AND ANNE E. WACHENFELD. *Effect of physiologic acids on electrolytes in rat diaphragm.* Am. J. Physiol. 193(3): 623-626, 1958. The effects of physiologic acids on muscle electrolytes were studied by incubating rat hemidiaphragms in Krebs-Ringer solution or blood at pH 7.4 and 6.8. With the addition of acetic, lactic or beta hydroxybutyric acid to depress the pH, the sodium and potassium contents of muscles incubated in Krebs-Ringer solution were the same at each pH. This is in contrast to the efflux of these cations previously observed on adding hydrochloric acid to this medium. Using heparinized rat blood as the medium, the effect of hydrochloric acid was the same as in Krebs-Ringer solution. With acetic acid however, the pattern was reversed. Muscles in blood acidified with acetic acid actually retained more potassium than the controls at pH 7.4. An increased P_{CO_2} to depress the pH in blood did not affect the potassium content, but there was a gain of sodium in the muscles in the more acid blood so that the net cation content increased. In contrast to the physiologic acids which can penetrate the cells as intact molecules, hydrochloric acid produces an essentially extracellular acidosis. Hydrochloric acid therefore causes a displacement of intracellular cations as hydrogen ions penetrate the cell. With blood, which is better buffered than the intracellular fluid, acetic acid and carbon dioxide depress the intracellular pH more than the extracellular pH. The pH gradient across the membrane is then analogous to that with hydrochloric acid but in the opposite direction resulting in a movement of cations into the cells.

THE CALCULATIONS of Cullen and Van Slyke (1) showed that a greater proportion of an acid load is buffered in the extravascular fluid than can be accounted for by its intrinsic buffer content. A previous publication from our laboratory (2) reviewed the possible role of intracellular cations in buffering an extracellular acidosis. Rat diaphragms incubated in acid media lost more sodium and potassium than their controls at pH 7.4 and the extent of this loss was increased by further depression of the pH. Earlier work on frog muscles by Fenn and Cobb (3) showed the dependence of muscle potassium on the extracellular pH. These results are consistent with some *in vivo* observations. Swan and Pitts (4) and Tobin (5) infused hydrochloric acid into intact animals and observed changes in the plasma sodium and potassium which suggest that tissue cells lose cations in an extracellular acidosis and that this partially buffers the acid load. In these experiments (2, 4, 5), the acidosis was induced by the addition of hydrochloric acid to the extracellular fluid.

With the exception of an occasional ammonium chloride excess, this kind of acidosis is only seen in the laboratory. The acid loads to which an animal is normally subject are due to accumulations of keto-acids in diabetes or fasting, of lactic acid in fatigue, or of carbonic acid in hypercapnia. These acids are not only capable of entering the cells as undissociated molecules, but are in fact produced inside the cells. The changes in intracellular electrolyte concentrations in the presence of these more physiologic acids have been investigated.

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EXPERIMENTAL

As previously described (2), hemidiaphragms from rats weighing 150 ± 5 gm were incubated for 40 minutes at 38°C in the appropriate medium in an Erlenmeyer flask shaken at 100 min. The nutrient medium was a Krebs-Ringer solution containing 1 gm./l. of glucose and 200 U./l. of insulin. The buffer used was bicarbonate and carbon dioxide. Except in those experiments in which the partial pressure of carbon dioxide was itself the variable, 5% carbon dioxide in oxygen was used. Usually, one hemidiaphragm was incubated at pH 7.4 and the other at 6.8 or some intermediate pH.

In some experiments the hemidiaphragms were incubated in whole rat blood. Rats weighing > 300 gm were anesthetized with sodium pentobarbital and exsanguinated with a syringe and needle in the abdominal aorta. The diaphragms were taken from other rats of the usual size.

Muscle electrolyte concentrations were determined by flame photometric analysis of a nitric acid digest of the muscle.

RESULTS AND DISCUSSION

Metabolic Acidosis in Krebs-Ringer Solutions. In some preliminary experiments, beta-hydroxybutyric acid was added to the nutrient medium to give a pH of 6.8. Muscles incubated in that medium did not lose more potassium or sodium than controls at pH 7.4 as was the case when hydrochloric acid was added. In another experiment, two solutions were prepared, both with pH 6.8. In one, this was achieved by adding hydrochloric acid and in the other by adding beta hydroxybutyric acid. The muscles incubated in the solution with the organic acid retained more potassium than those in the hydrochloric acid solution. The outcome was similar when muscles were incubated in solutions with and without beta-hydroxybutyric acid but where both were at pH 7.4. The results of these preliminary observations are summarized in table 1.

It is conceivable that the presence of beta-hydroxybutyric acid, a metabolite, might improve the energy metabolism of the muscles so that the potassium loss due to the trauma of *in vitro* preparation is reduced. Therefore, in all subsequent experiments with metabolic acids the solutions at each pH were adjusted to

TABLE 1. DIFFERENCES OF ELECTROLYTE CONCENTRATIONS DUE TO ADDITION OF β-HYRDOXYBUTYRIC ACID TO MEDIUM

Control Medium	Experimental Medium	ΔK	ΔNa	No. of Pairs
K-R pH 7.4	K-R + β OH butyric pH 6.8	1.0±1.1	−2.2±3.5	[illegible]
K-R + HCl pH 6.8	K-R + β OH butyric pH 6.8	4.5±2.2	1.1±2.0	[illegible]
K-R pH 7.4	K-R + β-OH butyric pH 7.4	2.6±1.5	−3.7±2.1	[illegible]

K-R = Krebs Ringer solution.

ΔK and ΔNa indicate experimental-control values. Therefore a negative sign indicates a smaller content of that electrolyte in the experimental muscles. Values are mEq/kg wet tissue ± E.

contain the same concentration of the acid anion concerned. Thus, when lactic acid was added to a solution to lower the pH to 6.8, a comparable amount of sodium lactate was added to the control solution at pH 7.4. Experiments of this type were carried out using beta hydroxybutyric acid, lactic acid and acetic acid. The results are summarized in table 2 along with the previously reported results using hydrochloric acid (2).

It will be noted that with the metabolic acids there is no loss of sodium or potassium comparable with that using hydrochloric acid. In spite of the equal concentrations of the acid anion at each pH, the results are consistent with those in the preliminary experiments where the metabolic acid molecule was added only to the solution at the lower pH. Where the concentration of the acid anion is the same at each pH, the important variables are the hydrogen ion concentration and the concentration of the undissociated acid. The latter is greater in the more acid solution. As the difference of the hydrogen ion concentration is the same as in the hydrochloric acid experiments, we may therefore conclude that the different effect of metabolic acids on the intracellular cations is due to the presence of their undissociated molecules. As these acids are metabolites they penetrate the cells, and at equilibrium their concentration inside the cells is presumably similar to that outside. This would depress the intracellular pH, but as the acids can enter as intact molecules, the extra hydrogen ions are accompanied by anions and so do not displace intracellular cations. In the case of hydrochloric acid, the entry of hydrogen

ions unaccompanied by chloride ions lowers the intracellular pH in the same way, but but causes an expulsion of cations.

Metabolic Acids in Whole Blood. Acetic acid and sodium acetate were added to aliquots of whole rat blood. When equilibrated with 5% carbon dioxide in oxygen, the pH of one was 6.8 and the other 7.2. After a 40 minute incubation period, the muscles incubated in the more acid blood contained 7.6 ± 1.3 mEq/kg more potassium than their controls. The sodium value showed no significant change. The comparable addition of hydrochloric acid to whole blood caused an efflux of potassium from the muscle cells similar to that seen when hydrochloric acid is added to Krebs-Ringer solution (table 3).

On the basis of some experiments with frog muscle, Fenn (6) has proposed that the intracellular fluid is less well buffered than the blood. Therefore, if the acetic acid concentration is the same inside and outside the cell, the pH would fall lower in the intracellular fluid than in the better buffered blood outside the cells. This would cause a pH gradient across the membrane analagous to that caused by hydrochloric acid but in the opposite direction. The result would be a movement of cation into the cells to restore equilibrium. This effect is not observed with acetic acid in Krebs-Ringer solution because that medium is not better buffered than the intracellular fluid. In the intact animal, the extracellular fluid is in rapid equilibrium with the blood so that in this respect it is as if the cells were surrounded by whole blood. Tobin (manuscript in preparation) has infused metabolic acids into intact rats and finds plasma electrolyte changes generally consistent with these *in vitro* observations and with the theory advanced by Fenn.

TABLE 2. DIFFERENCES OF ELECTROLYTE CONTENTS OF MUSCLES ON ADDING VARIOUS ACIDS TO KREBS-RINGER SOLUTION

Added to K-R Medium	ΔK	ΔNa	No. of Pairs
Acetic acid	−0.1±2.3	−2.5±2.8	12
β-OH butyric	−0.3±1.4	−0.8±2.2	12
Lactic acid	0.0±1.2	−0.7±3.7	17
HCl	−5.0±1.0	−3.3±2.4	20

In all experiments, the control medium is at pH 7.1 and contains the same concentration of the acid anion as the experimental medium which is at pH 6.8.
Values as in table 1.

TABLE 3. DIFFERENCES OF ELECTROLYTE CONTENTS OF MUSCLES INCUBATED IN WHOLE RAT BLOOD IN THE PRESENCE OF VARIOUS ACIDS

Added to Blood	ΔK	ΔNa	No. of Pairs
Acetic acid	7.6±1.3	−0.9±3.3	11
Carbon dioxide 2% vs. 10%	0.1±2.1	7.2±2.6	12
Hydrochloric acid	−5.2±2.4	−6.6±5.0	13

Values are as in table 1.

Carbon Dioxide. The acid load most commonly encountered by an animal results from an accumulation of carbon dioxide. This too is a 'penetrating' acid in the sense that lactic and acetic acids are and a similar effect on intracellular electrolytes might be expected. Cooke *et al.* (7) found an elevation of muscle potassium in rats subjected to chronic respiratory acidosis and Fenn and Asmus (8) found a transient fall in plasma K in cats in acute hypercapnia. In our *in vitro* experiments, the similarity of the effect is only partial.

Krebs-Ringer solution equilibrated with 5% and 10% carbon dioxide had a pH of 7.4 and 7.1, respectively. The muscles incubated at the lower pH lost potassium to about the same extent as was seen in experiments with hydrochloric acid. In whole blood, diaphragms incubated at a higher carbon dioxide tension did not lose more potassium than their controls, but they did not show the gain of potassium observed with acetic acid in whole blood. Their sodium contents however were higher than in the controls so that the total cation content was greater in the muscles incubated in the more acid blood. In these experiments, pH 7.4 and 7.1 were achieved using 2% and 10% carbon dioxide in oxygen. Preliminary experiments using 5% and 20% carbon dioxide had similar results.

Although there is no potassium accumulation in muscles incubated in whole blood at a higher carbon dioxide tension, there is a net gain of cation similar to that observed when acetic acid is added to the blood. With hydrochloric acid there is a net loss of cation from muscles in the more acid blood. The different effect of carbon dioxide can perhaps be attribu-

ated to its pharmacological actions independent of a pH effect. An increase in membrane permeability at higher tensions of carbon dioxide would cause a greater leakage of potassium and a higher influx of sodium. Our results with increased carbon dioxide tensions in whole blood are what would be expected if this leakage effect were superimposed on the type of cation accumulation observed when acetic acid is added to whole blood.

INTERPRETATION

The interpretation of these results is subject to the usual reservations applicable to *in vitro* experiments. The intact and healthy animal may be regarded as an open system in which disturbances of the anion-cation balance are dealt with by an array of physiological mechanisms which eventually excrete the excess acid or base. The tissues *in vitro* are in a closed system which may be compared with an animal without the renal or respiratory regulatory mechanisms. This is the extreme state. Intermediate between this and the state of the healthy animal is the situation in certain types of disease where there is impairment of regulatory functions to various degrees.

It is proposed therefore that these *in vitro* experiments demonstrate the underlying biophysical response of the muscle cells to acidosis and that the actual response *in vivo* is subject to the superimposition of physiological reactions, renal or otherwise.

We express our thanks to Dr. W. O. Fenn for valuable guidance in carrying out these experiments and in the preparation of the manuscript. In addition thanks are due to Mrs. Maria Dvorak for technical assistance.

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THE BIOSYNTHESIS OF MILK-FAT IN THE INTACT COW*

by

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INTRODUCTION

Recent publications from our laboratory have been concerned with the roles of the short-chain fatty acids, carbonate and glucose as precursors of milk-constituents[1-5]. These have been studied by the intravenous injection of the various metabolites appropriately labeled with ^{14}C.

The appreciation of the importance of the short-chain fatty acids in ruminant digestion[6] has served to focus attention on the nature of fat synthesis in ruminants. The milk triglycerides of these animals are characterized by their content of short-chain fatty acids. Reports by POPJAK, FOLLEY, FRENCH *et al.* have thrown much light on the roles of acetate and glucose in ruminant fat synthesis, and this work is extensively reviewed by POPJAK[11] and FOLLEY[12]. Some of their work was conducted on intact goats, but most of the data are from perfused udder and tissue-slice studies. The present report supplements previous knowledge of the utilization of acetate and glucose by observations made on the intact cow. In addition, it provides some information about fat synthesis from carbonate, formate, propionate and butyrate.

METHODS

The equipment and techniques for the conduction of tracer trials have been described by KLEIBER AND ROGERS[1]. Following the injection of a ^{14}C-labeled metabolite, continuous samples of respired carbon dioxide were taken for the first 3 hours and then for short periods at intervals up to 48 hours. Blood samples were taken at half hour intervals during the first 3 hours, and subsequently at milking times which were 8, 10, 24, 34 and 36 hours after the injection.

In the preliminary survey, the radio-activity of the fat constituents was determined following the injection of each of the metabolites listed in Table I. The table also gives the dosage injected and characteristics of the cows which were used. Individual tracer trials are identified by the name of the metabolite injected, followed by a Roman numeral which indicates the number of the trial in the series carried out with that metabolite.

The fats were separated from each of the milk-samples by the method of ROHSE AND GOULDING[13]. Four crude fractions were separated from each fat sample. These were the glycerol, the water-soluble non-volatile fatty acids, the water-insoluble steam volatile fatty acids, and the non-volatile fatty acids. The latter three fractions are each composed chiefly of the C_4, the C_6-C_{10} and the C_{12}-C_{20} fatty acids respectively. The fractionation procedure is outlined below.

1. 100 g of fat were saponified for 30 minutes with methanolic potassium hydroxide.
2. The non-saponifiable fraction was extracted with ether and discarded.
3. The soap solution was acidified with sulfuric acid and steam-distilled for 2 hours.

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TABLE I

Trial No.	Body weight (kg)	Daily fat production (g)	Position of label	Dosage (μc/kg)
Carbonate VI	484	542	1-C	20.70
Carbonate XIV	443	576	1-C	23.39
Glucose II	526	971	U-C	2.76
Acetate VII	609	525	1-C	5.72
Acetate X	479	433	2-C	8.20
Propionate II	494	384	1-C	9.31
Propionate IV	542	336	2-C	8.86
Butyrate II	517	302	1-C	7.39
Butyrate IV	459	783	2-C	11.10
Formate II	454	593	1-C	11.0

4. The non-volatile fatty acids were removed from the residue by filtration and ether extraction.

5. The residue was then neutralized with potassium hydroxide and evaporated to dryness. The glycerol was separated from the potassium sulfate crystals with methanol.

6. The steam-distillate was similarly neutralized and evaporated to dryness. It was then redissolved in 10 ml of water, and when this was acidified, the water-insoluble volatile fatty acids came out of solution. They were separated by filtration at 4°.

7. The radio-activity of the fat constituents was measured in the fashion described by KLEIBER *et al.* A small amount of each sample was combusted and the resultant carbon dioxide was trapped in barium hydroxide solution, which precipitated barium carbonate. This was planchetted and counted at infinite thickness in a flow-gas counter.

RESULTS

The results are summarized in Table II. The radio-activity of each sample was determined as the "Standardized Specific Activity", of which the units are μc/gram atom carbon: μc injected/kg body weight. By dividing the specific activity by the administered dosage, it is possible to compare results from experiments in which different dosages were injected into animals of different weights. Owing to the different

TABLE II

SUMMARY OF DATA FROM ALL TRIALS

Trial	Position of label	Σ_0^{48} I_t for			% of activity derived via carbonate feed			% of dose appearing in each of				$\int_0^{48}$ as % Resp. CO_2
		Lactose*	Glycerol	Fatty acids	Lactose*	Glycerol	Fatty acids	Lactose*	Glycerol	Fatty acids	Total* milk	
Carbonate VI	--	6.0	4.4	1.0	---	---	---	1.0	0.07	0.26	1.6	60.0
Carbonate XIV	--	0.7	6.6	0.6	---	---	---	1.3	0.14	0.18	2.0	87.1
Glucose II	Random	28.7	31.0	2.6	13.4	0.8	13.5	55.4	0.4	1.0	71.6	34.1
Acetate VII	1-C	10.3	5.0	25.3	86.0	100.0	3.0	2.0	0.1	4.6	6.8	74.6
Acetate X	2-C	20.5	30.2	26.3	25.5	13.4	2.4	3.4	0.4	4.6	9.0	62.8
Propionate II	1-C	23.5	26.7	6.6	54.6	30.0	16.0	3.9	0.3	1.0	7.0	109.9
Propionate IV	2-C	64.0	59.4	4.5	8.0	7.0	10.0	6.2	0.5	0.7	9.3	49.3
Butyrate II	1-C	15.1	8.3	10.6	56.1	62.0	7.0	2.0	0.7	1.2	4.7	70.6
Butyrate IV	2-C	27.7	25.3	7.2	22.6	16.0	7.0	6.1	0.8	2.9	17.2	52.1
Formate	--	20.5	0.4	9.5	22.1	30.0	75.0	4.0	0.2	0.16	12.4	37.7

* These values were determined in previously reported work, and are included in this table for comparison with the values for glycerol.

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rates of metabolic processes, the radio activity of a single sample is not the activity of that constituent as synthesized at that instant, but is rather the average radio-activity for the time-interval since the last milking. However, as milk-constituents are stored by the cow in the udder as soon as they are produced, and are thenceforth presumably metabolically inert, the radio-activity of the sample multiplied by the time interval between milkings gives the time-integral of the radio-activity. These integrals can be summated through a 48 hour period (at the end of which there is no further appreciable radio-activity in any milk-constituents). Thus, in Fig. 1, the straight line forming the top of each block is the average radio-activity of that milk-constituent during that period of time. The area of the block (down to the abscissa) is the time-integral of the radio-activity for that milking, and the sum of the areas of all the blocks is the total time integral. This last value is the best indication of the extent to which the milk constituent in question has been formed from the radio-active precursor. A dotted line in Fig. 1 is an estimate of the locus of the instantaneous activity of the glycerol moiety of the milk-fat. It should be noted however, that this is just one of a family of curves that could be drawn to fit the experimentally determined time-integrals. Similar graphs can be drawn of the results in each of the tracer trials conducted.

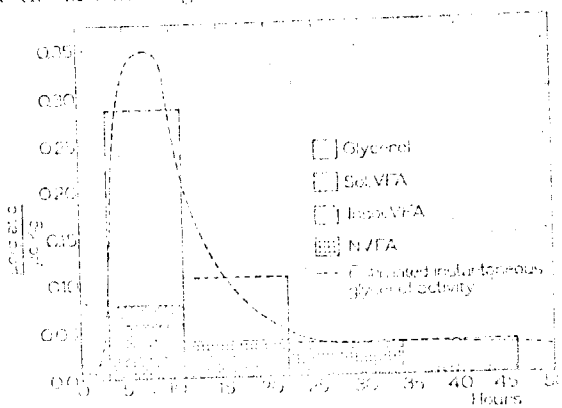

Fig. 1. Standardized specific activities following injection of ^{14}C bicarbonate.

Bicarbonate trials

It will be noted that the time-integrals of the radio-activities of the fat-moieties following the injection of $NaH^{14}CO_3$ were of a low order of magnitude (Table II). The results from these two trials are in close agreement, which suggests that the methods are reliable.

The extent of carbon dioxide fixation into any milk-constituent is conveniently expressed by KLEIBER[2] as the "Transfer Quotient"*. In all trials samples of respired carbon dioxide are taken throughout the experimental period and the standardized specific radio-activities can be plotted to give a smooth curve. Measurements have shown that the radio-activity of the respired carbon dioxide is the same as that of the plasma bicarbonate, which is assumed to be that of the tissue fluid in general. The curve is integrated over a 48 hour period, and similarly the radio-activity of each milk constituent is integrated over the same period. When the integral of the milk-constituent radio-activity is divided by that of the carbon dioxide, the quotient

* Transfer quotient "q" = $\frac{\sum_0^{48} \lambda s \, dt}{\int_0^{\infty} q s \, dt}$ where λs is the standardized specific activity of the milk-constituent, and qs is that for the respired carbon dioxide.

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"q", expresses the relative transfer of carbonate carbon to the milk-constituent concerned.

Calculations from Carbonate trials VI and XIV give transfer quotients of carbonate carbon to glycerol of 0.0725 and 0.0760 respectively. 7.4% is taken as the average. Once determined in this fashion, the transfer quotient can be applied to calculate the proportion of the activity in a milk-constituent which has come via the carbonate pool in trials where metabolites other than labeled carbonate have been injected. As before, the respired carbon dioxide activity is integrated over a 48 hour period, and this integral multiplied by "q" is divided by the integrated radio-activity of the milk-constituent,

i.e. $\frac{q \times \int_0^{48} qs \, dt}{\sum_0^{48} \lambda s \, dt}$ = transfer of carbonate carbon to milk constituent.

The values obtained from these calculations are listed in Table II.

The transfer quotient of carbonate carbon to the fatty acids of milk fat was 0.017 and 0.007 in Carbonate trials VI and XIV respectively. This indicates an average of about 1.0%, which is almost negligible.

Glucose trial

The glycerol following the injection of ^{14}C-labeled glucose was extremely radio-active, and was in fact the most radio-active compound isolated from the fats in this series of experiments. Conversely, the radio-activities of the fatty acids were very low. Table II shows that 71% of the injected isotope was recovered in the milk, which is greater by a factor of 4 than the isotope recovery in the milk following the injection of any of the other metabolites studied. The radio-activity of the respired carbon dioxide was correspondingly low.

Acetate trials

After the injection of either 1-^{14}C or 2-^{14}C labeled acetate, the percentage of the isotope appearing in the milk was low compared with that in the glucose trial, and the respired carbon dioxide radio-activity was correspondingly greater. The degree of labeling in the fatty acid moieties was about the same regardless of the position of the label on the injected acetate. It will be noted however, that the injection of 1-^{14}C-labeled acetate gave rise to practically no radio-activity in the glycerol, and that such radio-activity as was observed could be accounted for via the fixation of carbonate carbon. On the other hand, the 2-^{14}C-labeled acetate injection yielded glycerol as radio-active as the non-volatile fatty acid fraction.

Propionate trials

Our results show that, as might be expected, propionate is a potent precursor of glycerol. The time-integral of the specific radio-activity of the glycerol was higher than that of the fatty acids.

Butyrate trials

It is apparent that butyrate is intermediate between the extremes of lipogenic and glycogenic potentialities exhibited by acetate and propionate.

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Formate trial

Formate is a poor precursor of fatty acids, and 75% of the small activity observed in the latter after injection of labeled formate was derived via the carbonate pool. The glycerol radio-activity was considerably higher than that of the fatty acids, but still low when compared with the glycerol from all other trials except the Carbonate trials.

Delay in fat secretion

In each of the 6 trials, (with the exception of that with labeled glucose), the most highly radio-active fat obtained was that from the second milking (11 hours). This is in contrast with the casein and lactose, which always reached their highest radio-activity in the first milk sample (3 hours). In the case of the labeled glucose injection, the fat radio-activity was the same in the first and second samples.

DISCUSSION

Fatty acid synthesis

The potency of acetate as a milk-fat fatty acid precursor, and the large amount of acetate available to the cow, leave little doubt that it is quantitatively the main source of this moiety of the milk-fat. In our investigation, the time-integrals of the radio-activities of the milk-fat fatty acids after the injection of 1-^{14}C and 2-^{14}C-labeled acetate were about the same. Also, the percentage of the injected isotope which appeared in the fatty acids was the same in either case. These similarities suggest that acetate is utilized as a two-carbon unit for fatty acid synthesis, which is of course, consistent with the present concepts.

The radio-activity of the milk-fat fatty acids is smaller after injections of labeled butyrate than after those of correspondingly labeled acetate. That is to say, a smaller part of the blood butyrate than of acetate is used for fatty acid synthesis. In each milk-fat sample taken after labeled butyrate injections, the radio-activity of the soluble volatile fatty acids is higher than that of the insoluble volatile fatty acids in the same milk-fat sample (Table III). After acetate injections, however, the soluble volatile fatty acids are less radio-active than the insoluble volatile fatty acids. As

TABLE III

COMPARISON OF THE PATTERN OF LABELING IN THE THREE FATTY ACID FRACTIONS FROM SUCCESSIVE MILK-FAT SAMPLES*

Only the results after 1-^{14}C acetate and butyrate injections are compared here, but the pattern of labeling is essentially the same after the 2-^{14}C injections. The units are *pc/gram* atom C; *pc/kg* body weight.

Milk-sample number	^{14}C acetate injection			^{14}C butyrate injection		
	Sol.VFA	Insol.VFA	NVFA	Sol.VFA	Insol.VFA	NVFA
1	4.7	5.0	1.2	0.3	0.3	0.04
2	4.9	5.7	1.4	3.4	2.6	0.34
3	1.7	2.1	0.5	1.7	1.4	0.19
4	1.0	1.4	0.3	0.5	0.2	0.05
5	0.3	0.5	0.1	0.3	0.1	0.04
Σ% *to M*	61.0	77.8	17.8	53.2	38.0	5.7

* VFA = volatile fatty acids.
NVFA = non-volatile fatty acids.

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the soluble volatile fatty acid fraction is almost entirely butyric acid, it seems likely that some blood butyrate is incorporated directly into the butyric acid moiety of the milk-fat, and that not all blood butyrate is broken down into two-carbon units.

The similarity of the time-integrals of the standardized specific activities of the fatty acids after the injection of both 1-^{14}C and 2-^{14}C-labeled butyrate indicate that for most of the synthesis of fatty acids two carbon units are the smallest split products of butyrate.

The time-integrals of the standardized specific activities of the fatty acids from all trials listed in Table II, show that none of the other metabolites investigated is a potent precursor of milk-fat fatty acids. In particular, the low activity of the fatty acids following the injection of labeled glucose is in interesting contrast to the expected result in a non-ruminant. In a non-ruminant which is synthesising fat from a mainly non-fat diet, the necessary acetyl CoA units are derived from glucose, so that the injection of labeled glucose would give rise to highly labeled fatty acids in the fat[15]. In view of the fact that the cow absorbs little or no glucose from its gut[16], and that its blood glucose is synthesized from rumen fatty acids, it is hardly surprising that it does not use glucose as a source of fatty acids for milk-fat synthesis.

The level of radio-activity in the fatty acids after the injection of labeled propionate is similar to that after the injection of labeled glucose. There are undoubtedly pathways whereby propionate carbon is incorporated into fatty acids, but our data suggest that these are relatively unimportant in the cow. This is probably because of the large demand on blood propionate for glycogenesis.

The transfer quotient of carbonate carbon to fatty acids is only about 1%. This low value is of interest because a metabolic reaction of carbon dioxide with acetone to yield aceto-acetic acid has been demonstrated[17]. The aceto-acetate could be a precursor of fatty acids, but our results on carbonate transfer to fatty acids show that this pathway is either absent or negligible in the cow.

Formate too is a poor precursor of fatty acids, and can be considered as unimportant in this phase of the cow's metabolism.

Glycerol synthesis

DORSCHUK has studied glycerol metabolism in rats[18], and an accepted pathway of glycerol synthesis is that from dihydroxy-acetone phosphate produced in glycolysis. The extremely high glycerol radio-activity after the injection of labeled glucose into a cow suggests that a similar pathway is operative in this case. FOLLEY[19] found that ruminant mammary gland slices will synthesize fat from a substrate containing acetate as the sole organic constituent. The fat synthesis was enhanced by the addition of glucose, but only to the same extent as by the addition of glycerol. He also found that non-ruminant mammary gland slices would not synthesize fat from acetate, with or without glycerol, unless glucose was also present. FOLLEY's interpretation of these observations is that the main function of glucose in ruminant fat synthesis is as a precursor of glycerol, and that it is not required as a source of energy for the synthetic process. However, glucose cannot be regarded as a "Prime precursor", because in the cow, the glucose itself must be synthesised from fatty acids. Of the metabolites available from the rumen, our data show that propionate is the most potent precursor of glycerol. This observation concurs with that of KLEIBER *et al.*[5,7] and of BAXTER[8] regarding the synthesis of lactose.

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The injection of either labeled acetate or butyrate gave rise to radio-active glycerol. Therefore, all three of the principal short fatty acids are precursors of the glycerol moiety of the milk-fat. A glycogenic pathway in each of these three fatty acids seems to be via the tricarboxylic acid cycle. In each case, the ^{14}C activity in glycerol after the injection of the 1-^{14}C labeled fatty acid is much less than after the injection of the same fatty acid labeled in the 2-C position. This discrepancy would be expected of a fatty acid involved in the Krebs cycle, because a label on the carboxyl group of the injected fatty acid would appear on a terminal carbon of succinate, where it would be immediately vulnerable to oxidation. There is a greater chance of a 2-^{14}C label escaping the oxidation to CO_2 in the tricarboxylic acid cycle.

In view of the different glycerol radio-activities following the injections of 1-^{14}C and 2-^{14}C labeled butyrate, and of what is known about β-oxidation of fatty acids in general, it seems likely that some butyrate is split into two acetyl CoA units. These could be a source of glycerol and glucose via the tricarboxylic acid cycle when it is operating towards a net synthesis... as it appears to in the cow. However, as discussed by Kramer[32], labeled butyrate gives rise to lactose of greater activity than does labeled acetate, which suggests that butyrate may be a precursor of lactose without passing through the acetate pool.

The possibility of a glycogenic pathway for butyrate which does not pass through the acetate pool is further indicated by two observations related to the CO_2 expired[33].

1. The percentages of glycerol carbon that could be derived via the carbonate pool after the injection of 1-^{14}C and 2-^{14}C-labeled acetate were 100% and 67% respectively. The corresponding figures for butyrate injections are 62% and 16% (Table II).

2. As observed under *Fatty acid synthesis*, the relative radio-activities of the soluble and insoluble volatile fatty acid fractions of the milk fat after the injections of labeled butyrate suggest that some of the butyrate is not broken down to acetyl CoA units. If the only glycogenic pathway of butyrate were via the acetate pool then butyrate would be no more glycogenic than acetate; whereas in fact, it is.

The study of glycogenic from propionate has been a very active field. A pathway similar to that to lactate and pyruvate has been demonstrated in rats[34], but our results in the cow are not consistent with this pathway. If it were important in the cow, the glycerol (and lactose) radio-activities after the injection of 1-^{14}C and 2-^{14}C labeled propionate would be the same. Table II shows that this is not so. The main evidence for the rejection of this pathway in cow metabolism is provided by Brown's work on the fate of the carbon atoms from the propionate trials, discussed in this report. In the propionate trials, Brown found that the pattern of labeling in the alanine (which reflects that of pyruvate) was inconsistent with the acrylate pathway. A previously postulated pathway in which propionate enters the Krebs cycle via condensation with carbon dioxide to give succinate has been demonstrated by Flavin[35]. This pathway is consistent with our results, and would explain in part the large amount of carbon dioxide fixation observed in gluconeogenesis in the cow.

In the glycogenic fatty acids, acetate, propionic acid, butyrate, the fixation of carbon dioxide is an important factor, as is shown by the high transfer via carbonate pool listed in Table II. As these three fatty acids are quantitatively the most important products of the rumen's digestive system, it follows that carbon dioxide fixation

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is very important in the overall synthesis of the glycerol moiety of milk-fat.

The glycerol radio-activity following the injection of labeled formate is low (Table II). One of the known reactions of formate is that with glycine to yield serine[36]; serine can be deaminated to yield pyruvate[36], and then pyruvate is a source of glycerol. Franck[37] has found that the β carbon of serine (in the casein) is highly radio-active after the injection of labeled formate. Therefore in the cow, glycerol might be derived from formate via an amino acid. The degree of radio-activity in the glycerol after the injection of labeled formate, however, shows that this unusual pathway is quantitatively unimportant.

In most cases, the radio-activity of the glycerol was found to be the same as that of the lactose which had been determined in earlier work[7,8]. However, in two of the trials, acetate VII and butyrate II, the glycerol radio-activity time-integral was only about one-half of that of the lactose. Both of these trials involved the injection of a 1-^{14}C-labeled fatty acid, but there does not seem to be any ready explanation of the difference.

Secretion of milk-fat

Very little is known about the mechanism of milk-fat secretion, but the long-standing observation that the "strippings" from a cow have a higher than average fat-content would suggest that at least some of the fat is not secreted until the time of milking. It is not known whether this is the result of pressure conditions within the udder, or of some possible change in hormonal status when letting down the milk. The other theory that the "cream rose to the top" of the udder has been rejected on several grounds.

In our series of experiments, it has been strikingly apparent that fat is secreted with a greater delay than are the casein and lactose components. The delay is more than 5 hours and less than 10 hours, but the data do not permit an exact calculation of the length of the delay. The rapidity of fat synthesis *in vivo*[38] precludes the possibility that the delay is due to low reaction rates in the enzymic processes of synthesis. A comparison of the radio-activities of fat-components with those of other milk-constituents suggests that the fat obtained in the second milk-sample was actually synthesized during the first milking period. The delay in the peak of radio-activity is apparent in all of the fat constituents, including glycerol, and this shows that the delay occurs after esterification of the fatty acids to neutral fat. It is presumed therefore, that the fat is retained within the cells in which it is synthesized, and that the delay is a delay of the actual secretion into the lumen of the gland.

SUMMARY

The behavior of the principal metabolites of the cow as precursors of milk-fat has been studied. This has been carried out by determining the specific activities of the various fat-constituents after the injection of the metabolites labeled with ^{14}C. Significant fixation of carbon dioxide into the glycerol moiety was observed; that into fatty acids was almost negligible. Glucose is important in fat synthesis as a precursor of glycerol, but fatty acid synthesis from glucose is on a very small scale. Acetate is the most potent precursor of fatty acids, but it is also a source of glycerol. The transfer of acetate carbon to glycerol appears to be by way of the tricarboxylic acid cycle, through both the incorporation of acetyl CoA and by carbon dioxide fixation. Propionate is quite potent in fatty acid synthesis, but the ability of glycerol following the injection of labeled propionate suggests that propionate is the main carbohydrate precursor in the cow. Butyrate shows interaction lipogenic and glycogenic functions. Formate is not a source

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of fatty acids in milk-fat, but does contribute some carbon to glycerol. The peak radio activity in all of the fat-constituents was observed in the second milk-samples (taken at 11 hours), whereas for the casein and lactose it was in the first (3 hours after injection of the tracer).

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The effects of intraruminal infusions of acetic, propionic and butyric acids on the yield and composition of the milk of the cow

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In an earlier publication (Rook & Line, 1961) it was suggested that the increases in the yield and milk-fat (g.f.c.) content of milk that occur in response to an input of energy in the place of energy nutrition of the cow are due to increases in the rumen production of acetic and propionic acids. Evidence was given supporting the view that the acids differ in their effects on the synthesis of milk constituents by the mammary gland, acetic acid affecting synthesis of both lactose and proteins, propionic acid affecting specifically the synthesis of proteins.

The effect of the rumen production of the volatile fatty acids on the fat content of milk is already well established (Balch, Balch, Bartlett, Bertram, Johnson, Rowland & Turner, 1956). Unusually low fat contents occur when cows receive diets lacking in roughage, and these diets characteristically lead to a low ratio of acetic to propionic acid in the rumen. Balch & Rowland (1959) suggested that the low fat content is due to a low ruminal production of acetic acid on these diets but, as the synthesis of lactose (Reid, 1959), the amounts of propionic and butyric acids produced in the rumen of a cow may be important in determining the extent of fat synthesis in the mammary gland.

This paper describes three experiments to determine, with cows, the effects of continuous intraruminal infusions of acetic, propionic and butyric acids on the yield and composition of milk. Success was dependent on the development of a technique for the daily addition to the rumen of amounts of acids with a calorific value of not less than 1000 kcal, which is roughly equivalent to that of the smallest quantity of supplementary concentrate known from previous work (Rook & Line, 1961) to produce significant changes in the yield and composition of milk. Previously volatile fatty acids have been added to the diets of dairy cattle as sodium salts of the acids, either mixed with the concentrate part of the diet or as a solution, sometimes in admixture with small quantities of acetic acid, given directly through a fistula into the rumen (Balch & Rowland, 1959; Tyznik & Allen, 1951). In this way daily additions of up to 1 lb sodium acetate (the equivalent as acetic acid would provide 1600 kcal) have been reported, but larger quantities have produced harmful physiological effects (unpublished observations, probably resulting from the uptake of large amounts of sodium. A technique of infusing the acids in dilute aqueous solution directly into the rumen through a fistula suitable for continuous use over periods of several months was therefore developed in collaboration with Mr R. C. Campling of this Institute.

EXPERIMENTAL

Animals and their management. Lactating Friesian cows with large permanent rumen fistulas were used in the three experiments. They were housed in an experimental byre and the management routine was normal.

Experimental design. The order of the treatments, details of which are given in Table 1, followed a Latin square design in each experiment.

Table 1. *Details of experimental design*

Expt no.	No. of cows	Period of experiment (months of lactation)	Details of Latin square	Duration of each treatment (days)	Treatment: Infusion given daily	Treatment: Calories supplied (kcal/day)
1	3*	2nd–4th	3 × 3	28	(a) 10 gal water	—
					(b) 900 ml acetic acid + 10 gal water	3700
					(c) 900 ml propionic acid + 10 gal water	4150
2	3*	5th–8th	3 × 3	28	(a) 15 gal water	—
					(b) 1500 ml acetic acid + 15 gal water	5300
					(c) 1500 ml propionic acid + 15 gal water	7150
3	4	2nd–6th	Balanced 4 × 4	28	(a) 15 gal water	—
					(b) 1450 ml acetic acid + 15 gal water	5250
					(c) 1061 ml propionic acid + 15 gal water	5450
					(d) 915 ml butyric acid + 15 gal water	5350

* The same cows were used in Expts 1 and 2 but the order of treatments was reversed.

Feeds. The daily basal diet consisted of hay (16–18 lb/cow) and dairy concentrate cubes (barley 17, maize 20, wheat bran 20, decorticated groundnut meal 15, copra cake 10, palm-kernel cake 5, molasses 10, dicalcium phosphate 1, calcium carbonate 1, and salt 1%, with 5×10^6 i.u. vitamin A and 1×10^6 i.u. vitamin D added per ton) given at a rate of 1 lb/gal in Expts 1 and 2 and at 3·3 lb/gal in Expt 3, according to the milk yield of a cow at the beginning of an experiment. The daily amount of cubes was decreased by 2 lb/cow at the end of each experimental period in Expt 1 and by 1·5 lb in Expt 3.

Treatments. The water and the diluted acids (see Table 1) were given as continuous intraruminal infusions. From the first introduction of an acid, the daily amount was increased gradually from 500 ml on the 1st day to the amounts shown in Table 1 on the 4th day. Each day at noon the supply of water or dilute acid for 24 h was introduced into a reservoir above each cow and was allowed to flow into the rumen through a rubber tube. The tube passed through the inflated rubber bung that closed the fistula to a metal cage covered by a silk bag and anchored, a few inches above the

Table 2. *Treatment mean values, with their standard errors, based on results for the last 8 days of each treatment period for each cow, for the yields and composition of milk and the yields of milk constituents*

Cow no.	Supplement	Yield (lb./day)	Fat %	Fat lb./day	Soluble-protein %	Soluble-protein lb./day	Lactose %	Lactose lb./day	Casein (N × 6·38) %	Casein (N × 6·38) lb./day	Casein mg. N/100 g.*	Casein lb./day	Soluble protein mg. N/100 g.*	Soluble protein lb./day	Non-protein N (mg./100 g.)*
1	None	22·7	3·42	0·75	0·72	1·61	4·77	1·05	3·22	0·65	360	0·32	—	—	—
	Acetic acid	25·7	3·55	0·92	0·63	2·15	4·78	1·19	2·99	0·72	353	0·36	—	—	—
	Propionic acid	24·7	3·59	0·76	0·94	2·15	4·87	1·17	3·16	0·75	376	0·38	—	—	—
	Sig. of differences between two means	±0·39	±0·17	±0·05	±0·09	±0·24	±0·02	±0·02	±0·08	±0·02	±10	±0·027	—	—	—
2	None	18·4	3·68	0·67	0·74	1·36	4·35	0·81	3·29	0·57	373	0·42	194	0·121	246
	Acetic acid	20·0	3·30	0·77	0·84	1·70	4·61	0·89	3·05	0·62	389	0·46	197	0·137	213
	Propionic acid	18·8	2·72	0·51	0·95	1·64	4·35	0·84	3·44	0·62	399	0·46	135	0·139	243
	Sig. of differences between two means	±0·46	±0·13	±0·03	±0·14	±0·06	±0·11	±0·04	±0·07	±0·02	±8	±0·016	±37	±0·025	±12
3	None	30·9	3·67	1·21	0·92	3·13	4·37	1·40	3·35	1·04	422	0·81	80	0·139	213
	Acetic acid	35·9	4·27	1·54	0·67	2·09	4·75	1·63	3·63	1·11	495	0·90	77	0·109	193
	Propionic acid	30·7	3·38	1·08	0·82	2·75	4·95	1·58	3·64	1·07	436	0·66	63	0·173	229
	Butyric acid	31·6	4·26	1·33	0·65	2·05	4·63	1·42	3·45	1·02	433	0·83	87	0·162	213
	Sig. of differences between two means	±0·69	±0·22	±0·07	±0·03	±0·09	±0·04	±0·05	±0·03	±0·04	±7	±0·036	±26	±0·027	±95

* Calculated on a fat-free basis.

dose of the acid, by a weight. The rate of flow was adjusted, either by the insertion of a piece of glass capillary in the rubber tube or by a metal screw clip, to deliver the contents of the reservoir into the rumen in about 20 h. During the infusions the animals drank little, if at all, and the total water intake was not markedly increased.

Sampling and methods of analysis. Milk yield was recorded, and samples of milk were taken at each milking during the three experiments and weighted composite samples for the successive 4-day periods were prepared for each cow. All the samples were analysed for fat and total solids and, in addition, samples for the last two 4-day periods of each treatment were analysed for lactose and total, non-casein and, in Expts 2 and 3, non-protein, nitrogen. The methods of analysis have been described previously (Rook & Line, 1961).

RESULTS

For the three experiments, the treatment mean values, with standard errors for the yield and composition of the milk, and the yields of milk constituents, over the last 8 days of each treatment period, are given in Table 2. The main changes with time, in all the cows in all the experiments, in the yield and composition of the milk after the administration of the supplements are presented graphically in Fig. 1.

Acetic-acid supplements

The acetic-acid supplements consistently caused an increase in the yield of milk, which was established progressively over a period of about 3 weeks, the mean increase ranging from 1·6 lb./cow/day in Expt 2 to 5·0 lb. in Expt 3, and in Expts 2 and 3 was highly significant ($P < 0·05$). The proportionate increases in yield in the three experiments were 13, 9 and 16%.

The supplements of acetic acid also increased the fat percentage in all experiments; the mean increase of 0·36 percentage units in Expt 3 was significant ($P < 0·05$), but the increases of 0·13 and 0·42 in Expts 1 and 2, respectively, did not reach significance ($P > 0·05$). An effect on fat percentage was observed in the first 4-day sample after the introduction of the supplement, and was fully established with the second 4-day sample (see Fig. 1). The effects on the percentages of S.N.F., lactose, protein (total N × 6·38) and of the protein fractions in the milk were in general small and not consistent from experiment to experiment. In Expt 3, however, a significant ($P < 0·05$) decrease in the percentage of protein and soluble protein N (non-casein N minus non-protein N) and a significant ($P < 0·05$) increase in the percentage of lactose were observed. Calculations of the percentages of non-protein N were made only in Expts 2 and 3, but the concentrations in milk were decreased by the acetic-acid supplement in both experiments and by a highly significant ($P < 0·01$) amount in Expt 3. This effect reflects a more efficient use of food N during the period of acetic-acid administration.

The daily yield of fat was increased by 18–27% by the acetic-acid supplements; these increases were about twice as great as those of 9–14% in the yields of milk, S.N.F., lactose and proteins.

Propionic-acid supplements

With the supplements of propionic acid there were no consistent changes in the yields of milk; the small apparent changes varied in size from experiment to experiment and in no instance reached significance ($P > 0.05$). But consistent effects were observed on the contents of fat, S.N.F. and protein. The mean values for fat percentage over the last 8 days of propionic-acid supplementation were lower than the control values by 0·32, 0·36 and 0·29 percentage units in Expts 1, 2 and 3 respectively, and the

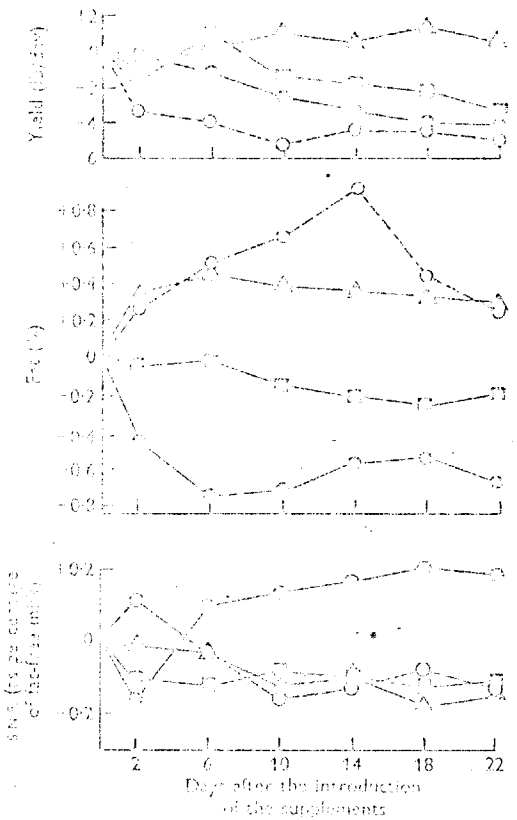

Fig. 1. Expts 1–3. Mean changes with time, for all the cows, in the yield and the fat and [illegible] contents of the milk after the introduction of intraruminal supplements of [illegible] butyric acids. □—□, no supplement; △—△, acetic-acid supplement; [illegible] propionic-acid supplement; ○—○, butyric-acid supplement. The results for the [illegible] supplement refer to Expt 3 only.

[illegible] significant ($P < 0.05$) in Expt 3. As with the acetic-acid supplements, the effect on fat percentage was immediate, but the most pronounced effect, a mean [illegible] of 0·7 percentage units, occurred in the second 4-day sample after the introduction of propionic acid, and thereafter there was a partial recovery.

The mean increases in S.N.F. percentage in Expts 1, 2 and 3 respectively, were

0·22, 0·21 and 0·30 percentage units, and in Expt 3 the effect was very highly significant ($P < 0.001$). In contrast to the effect on fat percentage, the change was not immediate but, as with the effect of acetic acid on milk yield, it was established progressively over a period of about 3 weeks. The increases in S.N.F. percentage were due largely to increases in the percentages of casein and the soluble protein, the proportionate increases in protein content in Expts 1, 2 and 3 being 5, 8 and [illegible] respectively. There was no effect on the non-protein N content of the milk, and the changes in lactose content were small and not significant ($P > 0.05$).

The propionic-acid supplements increased the yield of milk proteins by 6–17%, and decreased that of fat by up to 10%, there being no consistent effect on the yield of lactose.

Butyric-acid supplement

The supplement of butyric acid given in Expt 3 had no effect on the yield of milk but increased significantly the fat percentage ($P < 0.05$) by a mean value of 0·[illegible] over the last 8 days of the treatment period. This increase in fat content was equal to that obtained, in the same experiment, with an isocaloric supplement of acetic acid, but owing to the unchanged milk yield, the increase in the yield of fat, of 10%, was only one-third of that obtained with acetic acid. The effect of the butyric-acid supplement on fat percentage was at a peak, however, in the fourth 4-day sample after introduction of the supplement, showing a mean increase over the control value of [illegible], but thereafter the effect declined rapidly to less than half this value by the end of the treatment period. A significant ($P < 0.05$) increase in S.N.F. percentage of 0·16 was also obtained, but it was contributed to almost equally by increases in the lactose and protein contents which singly were not significantly increased ($P > 0.05$). There was no effect on the yield of S.N.F.

DISCUSSION

The characteristic effects of the intraruminal infusions of the individual steam-volatile fatty acids on the yield and fat and S.N.F. contents of milk are clearly shown in Fig. 1. Acetic acid had a general effect; it increased the yield of milk and the yields of all milk constituents, and also specifically increased the fat percentage. In contrast, propionic and butyric acids affected only the composition of milk: propionic acid decreased the fat percentage and increased the protein, and hence the S.N.F., percentage, and butyric acid increased the fat percentage. Whereas the effects on fat content were apparent in the first 4-day sample of milk after the introduction of the acids, the effects on protein and S.N.F. contents, and on the yield of milk and the associated yields of milk constituents, were progressive and not fully established until about the 3rd week of treatment.

In assessing the significance of these effects it is necessary to recognize that intraruminal additions of large amounts of organic acids may affect the fermentation of foodstuffs within the rumen. The distinct differences in the effects on the yield and composition of milk suggest, however, specific metabolic responses to the additions of the individual acids.

The differential effects of the individual volatile fatty acids on milk secretion could depend on the differences in sites of their metabolism. Of the three acids studied, a proportion of acetate or none of their total content is present in peripheral blood; propionate and butyrate, after absorption from the rumen, are metabolized almost wholly in the liver cells of the cow (see Annison, Hill & Lewis, 1959). It is probable, therefore, that the mammary gland is only affected by mammary tissue in any quantity as a source of carbon for the synthesis of fat, lactose and protein. Energy for synthetic reactions may be derived from phosphorylation, but in the lactating cow (Roch & Chase, 1956) and other ruminants (Reid, 1959), hence ATP and phosphate normally maintain blood concentration constant within narrow limits, and the supply to the mammary gland of ATP as utilized in the production of adenosinetriphosphate is likely to be more or less wholly dependent on the amount of acetate absorbed from the rumen than with the amounts of propionate or butyrate. It is reasonable, then, that the intraruminal addition of acetate should affect the rate of synthesis of all milk constituents, and the yield of milk, because the mammary secretion is maintained isotonic with blood plasma and an increase in the rate of synthesis of lactose will increase the amount of water in milk (see also Root & Wood, 1959). Since, with an increased supply of acetate from the rumen, the proportionate increases in the yields of fat and protein would be comparable to the same order, an intraruminal addition of acetate, according to this mechanism, be expected to give an increased milk yield with but little change in milk composition. The observed effects of intraruminal acetate and acetate supplement suggest, therefore, that there is no specific effect on fat synthesis. Acetate is known to be a precursor of fatty acids (Chaykin, 1959) and it is probable that the intraruminal addition of acetate increase incorporation of the acetate carbon, thereby, into the supply of acetate as precursor and thus the yield of milk fat.

Intraruminal butyric acid, from the rumen cannot directly affect the mammary gland. Annison & Hill (1957) have shown that intravenous injections of butyric acid, respectively, cause a decrease and increase in the concentration of total ketone bodies in the peripheral blood. The effects of the intraruminal addition of propionic and butyric acids on fat synthesis may be due to an effect on fat synthesis of the concentration of ketone bodies, since the liver is thought to be, like acetate, a precursor of fatty acids (Bailey, 1959; Shaw & Lakshmanan, 1957; Glascock, 1958). The increase in the concentration of ketone bodies after the first meal of the day of either propionic or butyric acid may result from an increase in their hepatic uptake, leading to a partial recovery of normal concentrations of ketone bodies in the plasma (cf. Thayer, Annison & Tepperman, 1958).

The effect (Reid, 1959) of the amount of propionate absorbed from the rumen on the overall demand for the synthesis of amino acids in the mammary gland, and hence the rate of synthesis of amino acids and hence the rate of synthesis of milk proteins are synthesized from the plasma amino acids (Reid, 1959). The precursor of lactose is the glucose of

the plasma (Malpress, 1958), but because of the effectiveness of the mechanism controlling plasma glucose concentration it is unlikely that any end-product of food digestion would influence the rate of lactose synthesis without, as with acetate, at the same time influencing the rates of synthesis of other milk constituents.

The distinct patterns of change in milk synthesis with the intraruminal additions of the individual volatile fatty acids are of particular biochemical importance since they have been established in the intact animal and presumably have developed from metabolic pathways that quantitatively are of importance in relation to milk synthesis. The experimental results reported have side implications also in practical dairy husbandry since, together with the knowledge of the effect of the cow's diet on the ruminal production of the volatile fatty acids (Balch & Rowland, 1957), they provide a rational basis and for advice in instances when, owing to unsuitable causes, milk is low in fat or S.N.F. It should now be possible by selection of foods to obtain for milking cows rations which, in terms of the ruminal production of volatile fatty acids, are favourable to high milk yield, high fat content or high S.N.F. content. Such considerations and the possibility of the use of the acids or their salts as supplements to rations for milking cows will be discussed more fully in subsequent publications.

SUMMARY

1. The effects on the yield and composition of the milk of the cow of intraruminal additions of dilute aqueous solutions of individual volatile fatty acids, as supplements to the basal diet, have been studied in three experiments involving a total of [illegible] cows. In the first two, infusions of acetic and propionic acids were compared, and in the third, infusions of acetic, propionic and butyric acids in all the experiments, infusion of water only was given as a control treatment.

2. A supplement of acetic acid caused an increase in milk yield and in the yields of fat, lactose and protein and a specific increase in fat percentage. Supplements of propionic or butyric acid had no effect on the yield of milk, but a propionic-acid supplement specifically increased the yield and percentage of fat and increased the yield and percentage of protein and solids-not-fat, while a butyric-acid supplement specifically increased the yield and percentage of fat.

3. These distinct differences in the effects of the individual volatile fatty acids on the synthesis in the mammary gland of the major constituents of milk are discussed in relation to current knowledge of the metabolism of acetate, propionate and butyrate in the ruminant.

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LACTOSE SYNTHESIS

I. THE DISTRIBUTION OF C^{14} IN LACTOSE OF MILK AFTER INTRAVENOUS INJECTION OF C^{14} COMPOUNDS*

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Glucose of the blood has been considered to be the major source of the carbon of lactose in milk, and recent studies with labeled glucose have substantiated this conclusion. Kleiber *et al.* (1) injected[1] glucose-U-C^{14} intravenously into a lactating cow and found approximately 50 per cent of the C^{14} converted to milk lactose. They calculated that about four-fifths of the lactose carbon originated from plasma glucose or a pool of metabolites which were rapidly interconverted with plasma glucose. Similar conclusions were reached by Reiss and Barry (2) from studies with a goat given glucose-C^{14}. According to previous evidence, the transformation of glucose to lactose may be considered to occur as follows:

(1) Glucose + ATP → G-6-P + ADP

(2) G-6-P ⇌ G-1-P

(3) G-1-P + UTP ⇌ UDPG + P-P

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[1] The following abbreviations are used: ADP for adenosine diphosphate; ATP for adenosine triphosphate; C-1, C-2, etc., for carbon of position 1, carbon of position 2, etc.; G-1-P for glucose 1 phosphate; G-6-P for glucose 6 phosphate; 6-PG oxidative pathway for 6 phosphogluconate oxidative pathway or direct oxidative pathway; glucose-U-C^{14} for uniformly C^{14}-labeled glucose; P for phosphate; P-P for pyrophosphate; UDP galactose for uridine diphosphate galactose; UDPG for uridine diphosphate glucose; UTP for uridine triphosphate.

(1) UTP + G-1-P → UDP galactose

(2) UDP galactose + G-1-P → lactose-1-P

(3) lactose-1-P → lactose + P

Gander *et al.* (8) provided evidence for Reaction 5, and Caputto and Trucco (4) and Smith and Ashwell (5) have isolated UDP(5) from mammary gland. Caputto and Trucco (4) and Maxwell *et al.* (6) have observed that galactokinase is present in mammary gland of rat and that Reaction 3 occurs.

Clearly, in this synthesis the two hexose moieties would be expected to [illegible] in identical isotopic patterns, since G-1-P is a precursor of both glucose and galactose. A number of investigators (7–10) have observed similar labeling of the hexoses. However, Schmidt (11) has reported that, when rabbits were given labeled acetate, pyruvate, lactate, or glucose, the (14)C distributions in the two hexoses of the milk lactose were not identical. The present communication describes an investigation of hexose formation in lactating rabbits and goats. It has been established that the galactose and glucose have distinctly different (14)C patterns. A hypothesis (Scheme No. 1, (7)) is given intended to explain the observed (14)C in glucose (C) was abnormal, the difference between the two hexoses was not large.

Methods

Preparation of (14)C-Compounds and Oxidation of Lactose from Milk—The procedure was that of Kleiber and collaborators (12, 13), with the exception of the [illegible] which was carried out as described by Lipmann *et al.* (14). Some of the quantities of the experiments are indicated in Table II. In certain experiments the hexose was purified by chromatography [illegible] (15). The combined fractions contain the lactose [illegible] concentrated to a small volume, and hot ethanol was then added to [illegible] percent concentration to induce crystallization.

Preparation of Glucose and Galactose—0.7 to 0.8 gm. of lactose was hydrolyzed [illegible] 15 ml. of 1 N H_2SO_4 for 5 hours in a boiling water bath [illegible] sulfuric acid. The sugar solution was [illegible] to a small volume, placed on a heavy lifting power clay and [illegible] column of 2 cm. The size was chosen on the basis of [illegible] (Whatman standard grade, cellulose, 20.5 inches [illegible] 1.5 liters of water, prepared by stripping the cellulose [illegible] with a small portion with a mixture as directed by [illegible] Three liters were prepared by mixing 3 parts of butanol [illegible] with 1 part of hydrochloric acid, and was pumped [illegible] at 25° at a rate of 1 ml. or less per minute and was

collected in 15 ml. fractions. The glucose began to appear when approximately 2 liters of solvent had passed through the column; an additional 1.5 liters separated both the glucose and galactose as slightly overlapping fractions. The column was regenerated by passing through it in succession 1 liter of H_2O, 1 liter of ethanol, and 1 liter of the butanol solvent. The sugar was determined by the anthrone reaction (17) upon aliquots of the eluate after evaporation of the solvent at 100°. The composition of the various "homogeneous" fractions of sugar was examined by chromatography on Whatman No. 1 paper with a solvent mixture of isopropyl alcohol and water (9:1, v/v); 100 γ of total sugar were placed on each spot (18, 19). About ten consecutive fractions were combined. Pure crystalline sugar fractions were combined without addition of water, and the solvent was evaporated to about 5 to 10 ml., at which time crystallization usually began. In some cases glucose did not crystallize by this procedure. In these instances it was evaporated to dryness, dissolved in boiling absolute ethanol, and then concentrated to about 3 to 5 ml. when crystallization occurred. The melting point of the galactose was 165–168° and of the glucose 145–147°. About 125 gm. each of glucose and galactose was recovered.

Degradation of Sugars with Leuconostoc mesenteroides—The sugars were degraded with a suspension of cells as described by Bernstein *et al.* (20) except for the following modifications. The fermentations were conducted in 50-ml. Warburg flasks, and the CO_2 evolved from C-1 was absorbed with nitrite and measured quantitative CO_2 absorber containing 3.7 N NaOH. The progress of the fermentation was determined by the [illegible] of CO_2 evolved. The lactic acid was chromatographed on Celite and was oxidized with MnO_4 to CO_2 and acetaldehyde. The acetaldehyde was oxidized with dichromate to acetic acid by the same procedure used for the ethanol oxidation. Acetic acid was degraded by the Schmidt procedure (21).

Cells used for the galactose degradation were adapted to this sugar by serial transfer on media in which the glucose was gradually replaced by galactose, and the culture thereafter was maintained on galactose medium.

Procedure Degradation of Gentiobenzimidazole—The synthesis and degradation of the gentiobenzimidazole were identical with that described for glucobenzimidazole (20) except that the 2-benzimidazolealdehyde was oxidized by permanganate to CO_2. This procedure yielded the average specific activity of C-1 and C-2 combined. C-3, C-4, and C-5 were obtained as formate and C-6 as formaldehyde.

Degradation of Sugars with Lactobacillus casei—The procedure was based on the degradation described by Wood *et al.* (22). Washed cells of *L. casei*, when grown on glucose, will ferment glucose but not galactose. *L.*

[illegible] and [illegible] lactobacillus, and in shed cells which have been [illegible] [illegible] will ferment galactose to lactic acid quantitatively. A [illegible] of glucose and galactose mixtures can therefore be accomplished [illegible] *L. casei* without separation of the sugars. The sugar mixture [illegible] in a 175 ml. Warburg flask with cells grown on glucose. [illegible] down strongly to a low level, the fermentation is [illegible] with [illegible]. The resulting lactic acid is [illegible] [illegible] in face the acid solution after removal of the cells by [illegible] [illegible]. The lactic acid derived from galactose is then degraded. The [illegible] of the ether extraction is freed from ether, neutralized, and the [illegible] fermented with gulac-acclimated *L. casei*. The residual [illegible] is extracted with ether and degraded. The method [illegible] that it is much more rapid than the *L. mesenteroides* [illegible], which requires separation of glucose and galactose since [illegible] of *L. mesenteroides*, even when conditioned to galactose, do ferment [illegible] only. However, the *L. casei* preparations possess the [illegible] of the strain are used in mixtures rather than [illegible] in the [illegible].

[illegible] of [illegible] radioactivity was measured by gas phase (II), [illegible] per milligram per milligram of carbon [illegible] compound. The average [illegible] of the activities [illegible] glucose, and galactose were determined by calculation (2a) [illegible] the specific activity of the CO_2 by the number of [illegible] [illegible].

EXPERIMENTAL AND DISCUSSION

The reliability of the *L. mesenteroides* degradation of glucose has been [illegible] (2b), but it was necessary to determine the reliability of [illegible] of galactose samples degraded. For this purpose a sample [illegible] of galactose was prepared by enzymatic hydrolysis of lactose [illegible] *L. casei* degradation with [illegible]. The results of the [illegible] and calculation with [illegible] are shown in Table I. Experiments 1 and 2 [illegible] that the average of the specific activities of the individual carbons [illegible] the *L. mesenteroides* degradation is in fair agreement with the [illegible] [illegible] (C-1 and C-2) agree closely, the activity [illegible] (C-3 and C-4) is low, and there is poor agreement in [illegible] (C-5 and C-6). The reason for this [illegible] is not clear. [illegible] [illegible] to have been [illegible] [illegible] synthesis. [illegible] fermented with *L. mesenteroides* (Experiment 3), the [illegible] [illegible] for C-1, the high [illegible] for C-4.

The degradation of lactose by *L. casei* involving selective fermentation of glucose and galactose has not been investigated extensively, but the

TABLE I

Comparison of L. mesenteroides Degradation of Galactose and Periodate Degradation of Galactobenzimidazole

The values represent the specific activity in counts per minute per micromole of C.

Experiment No.	Type of degradation	C-1	C-2	C-3	C-4	C-5	C-6
1	*L. mesenteroides*	2.30	4.24	24.1	30.6	0.55	1.22
Average		3.22		38.4			1.22
2	HIO_4 oxidation of galactobenzimidazole[1]	2.23		30.3			0.44
3	*L. mesenteroides* with galactose-1-C^{14}	183.0	1.45	0.10	0.77	0.41	0.88

[1] Calculation of Experiments 1 and 2 is from Experiment 2, Table III. Galactose-1-C^{14} of Experiment 3 contained 17.8 atom per mole.

TABLE II

Degree of Specific Activity of C^{14} Distribution in Lactose of Cow Milk after Intravenous Injection of Sodium-C^{14}, L. casei Degradation

Time	Specific activity of CO_2	Sugar	Specific activity on basis of 12.8 in 100			Specific activity of CO_2	Recovery of CO_2 of lactose in carbonate
			C-6	C-5	C-3		
hr.	c.p.m./mg.					c.p.m. per mole	per cent
0.5–2.1	11.8	Glucose	4.3	3.0	100	27.7	99.8
		Galactose	3.1	13.5	100	20.2	
3.1–9	12.3	Glucose	2.0	2.3	100	27.3	102.1
		Galactose	0.7	15.5	100	25.5	
9–22.3	23.1	Glucose	5.8	6.7	100	6.3	101.5
		Galactose	11.8	27.5	100	6.8	

Milk samples and lactose calculation are described in Table III, Experiment 3.

results indicate that it may not be completely reliable. Data from the *L. casei* degradations are shown in Table II. The *L. casei* and *L. mesenteroides* degradations may be compared, since the lactose from the 3.1 to 9 hour time interval of Table II and lactose from Experiment 3, Table III, are the same. The specific activity for C-1, 6 for galactose is calculated to

[illegible] (C-2,4 as 100) in the *L. acidophilus* degradation (Table [illegible]) [illegible] raised to 0.7 by the *L. casei* procedure (Table II), and for C-2,5 [illegible] raised to 15.5. It thus is apparent that there is a considerable difference between the two degradations. All degradations other than those of Table II were therefore carried out with *L. acidophilus*.

[illegible] experiments of Table II were performed to determine whether the distribution of lactose changes with time. Nicher *et al.* (18), in studying [illegible] in the milk constituents of cows, found that the ratio of [illegible] of the milk products to that of the CO_2 changed with [illegible] and that there were several different [illegible]. Lactose is perhaps the [illegible] in carbon-14 fixation in milk compounds. For example, one pathway might involve fixation of CO_2 via the [illegible] cycle in the mammary gland. Table I shows [illegible] pattern of C^{14} in lactose with time; i.e., the [illegible] C^{14} increases in the 1,6 and 2,5 positions in the galactose [illegible]. The [illegible] is not possible [illegible]. It is clear that as time [illegible] is an important [illegible] in the C^{14} [illegible] in lactose.

[illegible] Table III are from experiments in which [illegible] were injected and the distribution of the C^{14} was determined in glucose and galactose of the milk lactose. All the lactoses [illegible] in Table III were from milk except [illegible] of Experiment 2, which is [illegible]. [illegible] the [illegible] activities found were expressed as percent [illegible] of the total activity found in the lactose, and the [illegible] activity of C^{14} in the degradation was calculated [illegible] on the sum of the specific activities of the individual carbons and [illegible] specific activity of the compound as determined by calculation. [illegible] ranged from 91.9 to 107.3 per cent and is considered within [illegible] error, which was possible even after the galactose degradation [illegible] Experiment 1, Table III. The sum of the activities of the glucose and [illegible] was equal to that of the lactose, indicating that the sums were [illegible]

[illegible] expected that the specific activities of the glucose and galactose would be equal. This finding is in agreement with the result [illegible] (2, 5, 7, 16). Thus it might be considered that [illegible] and galactose and hence lactose are synthesized [illegible] by [illegible] mechanisms. However, the distribution [illegible] of the glucose differed from that of the galactose, particularly [illegible] (Table [illegible]) are the reverse of [illegible]. Even if [illegible] could be made, the [illegible] such a difference and state that all [illegible] relate to a common precursor within the mammary gland for

the two monosaccharide moieties of lactose. In part, this conclusion was based upon a check of the pooling (the combined average specific activities

TABLE III

Distribution of C^{14} in Glucose (Glu) and Galactose (Gal) of Lactose of Milk L. acidophilus Degradation

Experiment	Lactose concentration	Specific activity of lactose	Lactose	Specific activity of lactose	Specific activity on basis of C-1 = 100							Recovery of C-1	Recovery of C-1
					C-1	C-2	C-3	C-4	C-5	C-6			
	gm/100 ml	μc/mg		μc/mg								μc/mg	%
1	CH_3COOH	128.7	Glu	64.5	4.0	4.0	73.7	100	1.4	3.0	33.6		96.8
			Gal	32.1	7.2	13.9	78.8	100	1.8	4.0	36.6		99.8
2	$CH_3CHOHCH_3$	231.6	Glu	297.0	3.2	4.4	78.2	100	2.3	2.4	32.8		94.0
			Gal	117.7	13.2	22.7	65.7	100	3.2	1.6	33.9		91.7
3	$NaHCO_3$	110.0	Glu	61.2	4.0	5.0	67.7	100	0.8	2.7	32.2		95.8
			Gal	27.0	17.0	31.1	66.2	100	3.0	3.0	29.4		99.1
4	$CH_3CHOHCH_3$	155.8	Glu	81.7	64	28	85	100	174	105	12.0		98.2
			Gal	51.2	86	65	87	100	167	161	12.6		91.9
5	$CH_3CH_2CH_2$	203.0	Glu	131.0	17.0	50	201	120	100	231	12.47		99.8
			Gal	278.0	7.2	0	215	154	201	54	701	14.27	100.3

[illegible] Experiment 1, [illegible] 13 hours after injecting 4.7 mc. of acetate-1-C^{14} [illegible] Experiment 2, [illegible] according to Keller *et al.* (12). Cow weighed [illegible] produced approximately 5 kilos of milk daily (Keller *et al.*, 25). Table II, [illegible] Experiment 3, [illegible] milk from first 6 lactate siblings [illegible] (Cole *et al.*, 3) [illegible] given intravenously to goat weighing [illegible] Lactose was [illegible] by Fuller *et al.* (26), purified on charcoal-Celite [illegible] 131-136 and 1-2 times with lactose [illegible] crystallized; Experiment 4, [illegible] 3, 4, 6, 8, and 13.2 hours after injecting 5 mc. of [illegible]-C^{14} intravenously. [illegible] from the 6 to 10 hour milk. Jersey cow weighed [illegible] and produced approximately 6 kilos of milk per day (Acetate, Trial [illegible]). Experiment 5, cow milked 1, 3, and 11 hours after injecting 0.86 mc. of acetate-2-[illegible] intravenously. Lactose crystallized (18) from the 3 to 11 hour sample, then diluted [illegible] times with lactose [illegible] and recrystallized. Jersey cow weighed 365 kilos [illegible] produced 7.6 kilos of milk per day (Acetate, Trial X). Experiment 5, cow milked 5 hours after injecting 1.75 mc. of glucose-1-C^{14} intravenously. Glucose and galactose [illegible] from milk sample, crystallized, and diluted 1:1 times lactose [illegible]. Specific activity is calculated on basis of alcohol dilution. Cow weighed 425 kilos and produced 9 kilos of milk per day (Glucose, Trial VII). The [illegible] have not been corrected for the above dilutions by lactose (C^{14}, glucose (C^{14}), or galactose (C^{14}). Designations in parentheses are those of the "Davis Tracer Team."

of C-1, C-2, and C-3, and also C-1, C-5, and C-6 of the two hexoses. However, it is seen from Table III that only through a degradation giving the individual carbons does the difference in the C^{14} pattern of the glucose and galactose become clearly evident.

[illegible] distribution in the glucose will be considered first. In Experiments [illegible], Table III, the distribution is unsymmetrical, C-3 having a lower [illegible] than C-1. This distribution is in contrast with the equal [illegible] from C-3 and C-4 of the glucose of liver glycogen when Na[illegible] was administered to fasted rats (20) and also to the equal labeling [illegible] found in urinary glucose of diabetic dogs given primitive[illegible] (27). However, conditions such as the nutritional state of the ani[illegible] influence the distribution of C^{14} (28, 29). Liver slices from fasted rats [illegible] the distribution of C^{14} in the glycogen in liver slices, but with [illegible] equally in C-3 and C-4 of the glycogen in liver slices, but with [illegible] C-3 had a lower activity than C-4 and the dif[illegible] was accentuated in the presence of glycerol (29).

[illegible] glucose moieties of the lactose samples of Experiments 1, 2, and 3, [illegible] the pattern of C^{14} patterns. These are what are expected to occur in the [illegible] of non-fasted animals, i.e., most of the activity is in C-3 [illegible] and C-3 have lower activity than C-1.

[illegible] and 3, Table III, [illegible] of the galactose of Experiments 1, 2, and 3, Table III, [illegible] the major difference between the glucose and galactose de[illegible] is in C-2 and C-3. The galactose has much more activity in [illegible] than in C-2. The decrease in activity from C-3 [illegible] the glucose. The distribution of C^{14} could be expected [illegible] through the 6-PG oxidative pathway and the [illegible] of this pathway in the distribution of the [illegible] by Wood *et al.* (30).

[illegible] Experiment 4, Table III, when acetate-2-C^{14} was used, both hexoses [illegible] activity. The activity is an important factor in [illegible] activity. In liver glycogen of fasted rats, 2.5 hours after [illegible] 2-C^{14} had been administered, Hixon *et al.* (31) found the specific [illegible] to be about 50 per cent higher for C-1,6 and C-2,5 than in the [illegible] C-3,4. However, in Sakami's experiments (32), when the time was 11 [illegible], the distribution was more nearly uniform. In the present experiment, the distribution would have been less uniform, if an earlier [illegible] of the milk had been degraded.

[illegible] Experiment 5, Table III, when glucose-1-C^{14} was the source of C^{14} [illegible] had a lower specific activity than the glucose (37% compared [illegible]). This observation is similar to that of other investi[illegible] (7, 27). The observation is further given glucose [illegible] the lactose from a cow given glucose [illegible] the activity of the [illegible] contained 37 per cent of the activity of the [illegible] Berger (2) observed a value of 66 per cent in goat lactose. [illegible]

[illegible] to communication, R. M. Butterworth, School of Veterinary Medicine, [illegible] of California, Davis, California.

a perfusion experiment with glucose-1-C^{14} Dinaut *et al.* (9) found 88.5 per cent. In contrast, when glucose-U-C^{14} was given, the specific activities of the glucose and galactose were found to be equal in goat lactose (Reiss and Berry (2)) and rabbit lactose (French *et al.* (7)). The different results with glucose-1-C^{14} and glucose-U-C^{14} may arise because more C-1 is lost through the 6-PG oxidative pathway during formation of the galactose moiety than during the formation of the glucose moiety.

A further test of the validity of the *L. mesenteroides* degradation has been obtained through Experiment 5, Table III. Butterworth has determined the C^{14} distribution by chemical degradation of a sample of lactose from an experiment similar to Experiment 5, Table III. He converted the glucose and galactose to gluconic and galactonic acids, which were oxidized with periodate. On the basis of the total activity of the hexose, Butterworth found the following distribution in per cent of the total labeling of the hexose: glucose C-1 = 94.5, C-6 = 1.5; galactose C-1 = 79.3, C-6 = 9.1. By *L. mesenteroides* degradation, the corresponding values (Table III) are glucose C-1 = 95.5, C-6 = 1.2, and galactose C-1 = 83, C-6 = 8.8. From these results it is clear not only that the galactose has a lower activity, but that its C^{14} is more widely distributed in the 6-carbon chain and that the two methods of degradation agree reasonably well. The presence of a probable explanation of the C^{14} distribution in sections of the galactose C-1 of both hexoses, although the C^{14} in C-6 might be due to the [illegible] than C-1 of both hexoses, although the C^{14} in C-6 might be due to the [illegible] activity. It seems unlikely that the C^{14} in C-3 is a result of CO_2 [illegible] because C-1 would likewise acquire equal C^{14} by this mechanism. [illegible] discussion of the randomization of C^{14} of hexose [illegible] (3) has pointed out that the reactions leading to the C-PG oxidative pathway. It is of interest that C-1 is randomized into other positions by these reactions.

It is apparent from Table III that the glucose moiety of the lactose has a distribution similar to that expected of the free blood glucose but differs from the galactose. With glucose-1-C^{14} the glucose moiety is almost exclusively labeled in C-1, and with acetate-1-C^{14} or $NaHCO_3$ it is labeled predominantly in C-3 and C-4. It is therefore proposed that the glucose moiety arises from the glucose which is in equilibrium with the blood glucose. In contrast it is proposed that the galactose moiety arises from hexose-1-phosphate which is formed in the mammary gland. The C^{14} distribution in the galactose is believed to reflect more directly the events occurring in the gland, and in general the C^{14} distribution is more complex in the galactose moiety than in the glucose. The possibility that known mechanisms of metabolism can explain the C^{14} distribution in the galactose will be considered by Wood *et al.* (30). Further evidence will also be presented that the glucose moiety is derived from free glucose.

SUMMARY

Milk lactose has been obtained from cows which were given intravenous acetate-1-C^{13}, $NaHCO^{13}O_3$, acetate-2-C^{14}, or glucose-1-C^{13} and from a goat which received acetate-1-C^{13}. The C^{13} distribution patterns in the glucose and galactose moieties have been determined by degradation with *Lactobacillus acidophilus* and compared. The following results have been obtained.

1. The glucose and galactose moieties of all lactoses examined approximately equal activities, but the distribution of the C^{13} differed in the two hexoses.

2. With acetate-1-C^{13} and $NaHC^{13}O_3$, the C-1 carbon of both hexoses contained the highest activity, but the C^{13} distribution was much less symmetrical in the galactose than in the glucose. In the galactose it was: C-1 > C-3 > C-2 > C-4 > C-5 or C-6. The C^{13} distribution in the glucose was similar to that expected to occur in the blood glucose.

3. With acetate-2-C^{14}, both sugars were similarly and quite uniformly labeled.

4. In galactose, C^{13} in the galactose had a lower activity than in the glucose and 5 per cent of the C^{13} was in positions other than C-1. These carbons contained only 3 per cent of the C^{13} in the glucose.

5. It is suggested that the glucose moiety of lactose may originate from blood glucose with little or no gluconeogenesis and the galactose from blood glucose by a pathway involving the mammary gland. A pathway of synthesis of the galactose moiety from the three-carbon precursor of the disaccharide lactose is indicated. This second pathway, which probably involves synthesis from small molecules, may account for only a small part of the galactose of the lactose.

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Metabolism of Labeled Ethanol, Acetate, Pyruvate, and Butyrate in "Drinker" and "Nondrinker" Rats*

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Segovia-Riquelme *et al.* (1) did not find any significant difference in the rate of conversion to CO_2 of the first carbon of ethanol between rats of the "drinker" and "nondrinker" strains. It was relevant to investigate whether a difference in the rate of oxidation of the second carbon of ethanol between both strains of rats could occur. Furthermore, since it has been proposed as a working hypothesis (2) that the metabolic difference between these two strains could be located in the rate of appearance of two carbon metabolites from carbohydrates, it was interesting to compare the rate of oxidation of the first, second, and third carbon atoms of pyruvate in rats belonging to both strains. To know whether other metabolic differences exist between these strains of rats, we have also studied the rate of oxidation of acetate labeled either in carbon 1 or carbon 2 and butyrate labeled in carbon 1.

EXPERIMENTAL PROCEDURE

The experiments were performed in albino rats of both sexes from the drinker and nondrinker strains of the Institute of Physical Education of the University of Chile, fed with a purified diet for more than 90 days before the experiments were started. The composition of the diet used was the same as reported by Mardones (Diet D) (3). The voluntary alcohol intake was measured daily for at least 1 month before the experiment was begun to be sure that the rats not only belonged to the respective strains under study, but also that they exhibited a drinker or nondrinker behavior. The range of the voluntary daily alcohol intake of the drinker rats was 0.24 to 0.83 ml of 95% ethanol per 100 g of body weight, and that of the nondrinker rats always less than 0.20 ml of 95% ethanol per 100 g of body weight. The rats were housed in individual cages where they had free access to two bottles containing, respectively, distilled water and a 10% (volume for volume) solution of 95% ethanol in distilled water. The consumptions of water and of ethanol solution were measured daily.

Ethanol-2-C^{14} was given at dosage levels of 8.4 or 33.6 mmoles per kg of body weight. Pyruvate labeled with C^{14} in carbon atoms 1, 2, or 3 was given in dosages of 0.5, 1, or 10 mmoles per kg of body weight. Acetate labeled either in carbon atoms 1 or 2 was given in dosages of 1.0 and 10.0 mmoles per kg of body weight. Butyrate labeled in carbon 1 was given in dosages of 0.5 and 10.0 mmoles per kg of body weight. All the acids were used in the form of sodium salts and given intraperitoneally, dissolved in 1 ml of distilled water per 100 g of body weight.

Immediately after the injection, the rats were placed in a respirometer. The exhaled CO_2 and its activity were measured hourly for 5 hours. The methods for collecting the CO_2 and for the measurement of its activity were the same as reported by Vitale *et al.* (4).

RESULTS

The results obtained in drinker and nondrinker rats were compared. The following items were measured: maximal rate of combustion (Table I), percentage of cumulative recovery of $C^{14}O_2$ at 5 hours after the injection (Table II), and shape of the curve of cumulative recovery (Figs. 1 to 4).

TABLE I

Maximal hourly rate of appearance of $C^{14}O_2$ in expired air of drinker and nondrinker rats receiving labeled metabolites

Labeled metabolite	Dose	Drinker		Nondrinker	
		No. of rats	$C^{14}O_2$*	No. of rats	$C^{14}O_2$
	mmole/kg		*mmole/kg*		*mmole/kg*
Ethanol-2-C^{14}	8.4	8	2.08 ± 0.30	8	2.52 ± 0.45
Ethanol-2-C^{14}	33.6	8	4.08 ± 0.76	8	3.28 ± 0.[illegible]
Acetate-1-C^{14}	1.0	9	0.41 ± 0.02	9	0.46 ± 0.[illegible]
Acetate-1-C^{14}	10.0	8	3.41 ± 0.27	8	3.21 ± 0.[illegible]
Acetate-2-C^{14}	1.0	4	0.21 ± 0.03	4	0.19 ± 0.[illegible]
Acetate-2-C^{14}	10.0	5	1.94 ± 0.12	5	1.92 ± 0.[illegible]
Pyruvate-1-C^{14}	0.5	4	0.31 ± 0.02	4	0.33 ± 0.[illegible]
Pyruvate-1-C^{14}	1.0	5	0.68 ± 0.02	3	0.77 ± 0.[illegible]
Pyruvate-1-C^{14}	10.0	12	5.22 ± 0.26	16	4.74 ± 0.[illegible]
Pyruvate-2-C^{14}	0.5	4	0.20 ± 0.02	4	0.20 ± 0.0[illegible]
Pyruvate-2-C^{14}	1.0	6	0.31 ± 0.03	6	0.33 ± 0.[illegible]
Pyruvate-2-C^{14}	10.0	11	1.01 ± 0.11	12	1.06 ± 0.1[illegible]
Pyruvate-3-C^{14}	0.5	3	0.11 ± 0.02	5	0.09 ± 0.0[illegible]
Pyruvate-3-C^{14}	10.0	8	0.42 ± 0.16	8	0.51 ± 0.[illegible]
Butyrate-1-C^{14}	0.5	4	0.21 ± 0.03	4	0.21 ± 0.[illegible]
Butyrate-1-C^{14}	10.0	4	2.0[illegible] ± 0.43	4	2.30 ± 0.[illegible]

* Mean ± standard error.

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[1] These terms have been defined previously (1). In general, they refer to rats that have a high voluntary alcohol intake and those that refuse alcohol offered to them.

All radioactive isotopes (Tracerlab) were obtained from Philips-Roxane, Netherlands.

TABLE II

Percentage of injected activity recovered at 5 hours as $C^{14}O_2$ in expired air of drinker and nondrinker rats receiving labeled metabolites

Labeled metabolite	Dose	Drinker		Nondrinker	
		No. of rats	Activity*	No. of rats	Activity*
	mmole/kg		%		%
Ethanol-2-C^{14}	8.4	8	69.9 ± 6.85	8	76.1 ± 4.05
Ethanol-2-C^{14}	33.6	8	55.5 ± 6.20	8	49.5 ± 2.60
Acetate-1-C^{14}	1.0	9	63.2 ± 2.75	9	61.7 ± 3.58
Acetate-1-C^{14}	10.0	8	62.5 ± 3.11	8	57.5 ± 3.52
Acetate-2-C^{14}	1.0	4	48.0 ± 3.15	4	48.0 ± 4.37
Acetate-2-C^{14}	10.0	5	51.2 ± 2.37	5	53.4 ± 2.97
Pyruvate-1-C^{14}	0.5	4	86.0 ± 4.82	4	88.0 ± 2.95
Pyruvate-1-C^{14}	1.0	5	97.6 ± 5.70	3	101.7 ± 2.34
Pyruvate-1-C^{14}	10.0	12	80.1 ± 2.40	16	80.3 ± 2.21
Pyruvate-2-C^{14}	0.5	4	81.5 ± 4.50	4	81.9 ± 3.75
Pyruvate-2-C^{14}	1.0	6	71.2 ± 1.88	6	72.0 ± 5.22
Pyruvate-2-C^{14}	10.0	11	25.8 ± 1.83	12	25.8 ± 2.59
Pyruvate-3-C^{14}	0.5	3	45.7 ± 3.49	5	43.6 ± 2.06
Pyruvate-3-C^{14}	10.0	8	15.1 ± 0.97	8	16.5 ± 1.64
Butyrate-1-C^{14}	0.5	4	60.5 ± 9.88	4	65.5 ± 3.06
Butyrate-1-C^{14}	10.0	4	56.8 ± 6.31	4	58.8 ± 2.46

* Mean ± standard error.

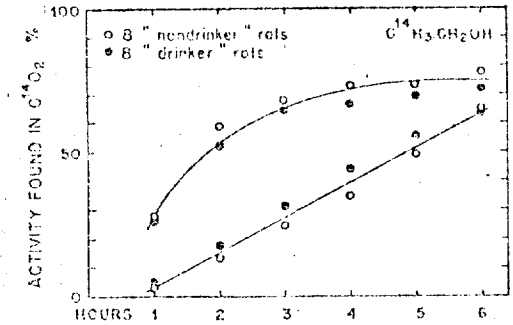

FIG. 1. Cumulative excretion of $C^{14}O_2$ by drinker and nondrinker rats inoculated intraperitoneally with ethanol labeled in carbon 2, in dosages of 8.4 (*upper curve*) and 33.6 (*lower curve*) mmoles per kg of body weight.

The experiments with ethanol-2-C^{14} (Fig. 1) showed that in both strains of rats, the $C^{14}O_2$ excretion curves followed the same pattern and no significant difference between drinker and nondrinker rats was observed. In the group receiving 33.6 mmoles of ethanol-2-C^{14} per kg, the cumulative recovery followed a straight line during the 6 experimental hours. The slope expressed in percentage of the injected activity per hour was 11.56 ± 0.87 in the drinker group and 11.20 ± 0.60 in the nondrinker one. The slope obtained by combining both groups was 11.38 ± 0.51, *i.e.* the average rate of oxidation of the second carbon of ethanol was 176 ± 7.9 mg of ethanol per kg of body weight per hour.

The experiments with labeled acetate (Fig. 2) showed no significant difference in the rate of oxidation of carbon 1 or carbon 2 between drinker and nondrinker rats at any of the dosages employed. The cumulative excretion of $C^{14}O_2$ was practically the same with high and low dosages in both strains, either for carbon 1 or carbon 2; but the rate of oxidation of carbon 2 was about one-half that of carbon 1.

The results obtained in experiments with rats receiving 0.5, 1, or 10 mmoles of labeled pyruvate per kg of body weight (Fig. 3) yielded evidence that no difference between the two groups of rats existed in the rates of oxidation of the first, the second, or the third carbon, at any dosage studied. The rate of cumulative excretion of $C^{14}O_2$ from the first carbon of pyruvate was very similar in the groups of rats receiving 0.5, 1, or 10 mmoles of pyruvate per kg of body weight. In contradistinction, the percentage of cumulative excretion of the $C^{14}O_2$ from the second carbon of pyruvate differed significantly with the dosage. Thus, the cumulative excretion of the $C^{14}O_2$ observed when the rats received pyruvate labeled in the second carbon was inversely related to the dosages. The same was observed for the oxidation of the third carbon.

The rate of appearance of $C^{14}O_2$ from butyrate labeled in carbon 1 (Fig. 4), was similar in drinker and nondrinker rats.

DISCUSSION

In the experiments reported, no metabolic differences between drinker and nondrinker rats that could explain their different appetites for ethanol were found. The lack of difference in the pyruvate metabolism in both strains of rats does not support the working hypothesis, which assumes that a blockade in the metabolic steps between three- and two-carbon metabolites could be the origin of the increased appetite for ethanol of drinker rats.

SUMMARY

No differences between drinker and nondrinker rats in the rate of appearance of $C^{14}O_2$ from ethanol labeled in carbon 2

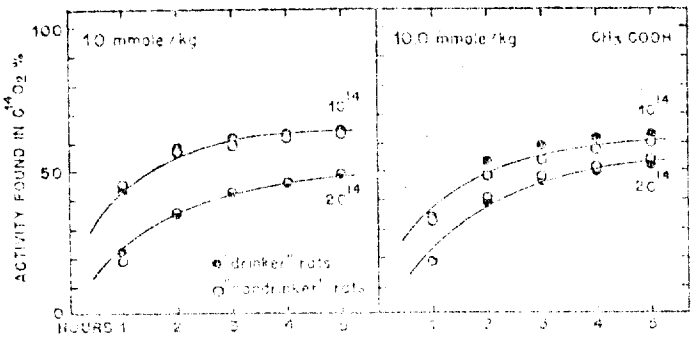

FIG. 2. Cumulative excretion of $C^{14}O_2$ by drinker and nondrinker rats inoculated intraperitoneally with acetate labeled in carbon 1 or carbon 2. *Left*, 1.0 mmole per kg of body weight; *right*, 10 mmoles per kg of body weight.

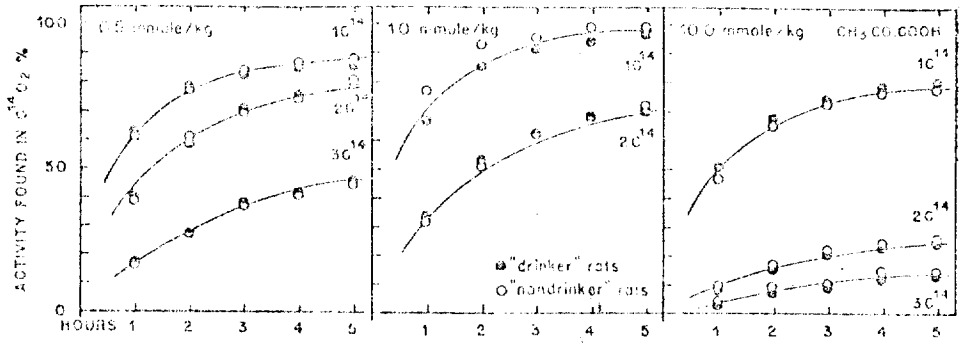

FIG. 3. Cumulative excretion of CO_2 by drinker and nondrinker rats inoculated intraperitoneally with pyruvate labeled in carbon atoms 1, 2 or 3, at three different dosage levels.

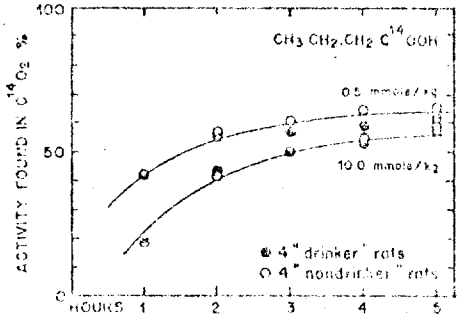

FIG. 4. Cumulative excretion of CO_2 by drinker and nondrinker rats inoculated intraperitoneally with butyrate labeled in carbon 1.

acetate labeled in carbon 1 or carbon 2, pyruvate labeled in carbon 1, carbon 2, or carbon 3, and butyrate labeled in carbon 1 were found for 5 hours after the intraperitoneal injection of these metabolites at several dosage levels.

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In Vivo Incorporation of Labeled Acetate into Liver and Serum Lipids of Rats on Different Dietary Regimens

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SERDARIEVICH, B., and CARROLL, K. K. *In vivo* incorporation of labeled acetate into liver and serum lipids of rats on different dietary regimens. Can. J. Biochem. 50, 557–562 (1972).

Incorporation of intravenously injected 1-^{14}C-acetate into lipids of liver and serum was studied in suckling rats and in rats fed commercial or semisynthetic diets containing no added fat, 15% butter, or 15% corn oil. Incorporation into liver cholesterol was higher on commercial than on semisynthetic diets and was stimulated by addition of corn oil to either diet. Incorporation into fatty acids did not parallel incorporation into cholesterol in the different dietary groups. Serum lipids tended to show a pattern of labeling similar to that of liver. Suckling rats showed low incorporation into both cholesterol and fatty acids. In general, these results correspond to the findings of earlier *in vitro* studies.

SERDARIEVICH, B., et CARROLL, K. K. *In vivo* incorporation of labeled acetate into liver and serum lipids of rats on different dietary regimens. Can. J. Biochem. 50, 557–562 (1972).

Après injection intraveineuse de 1-^{14}C-acétate, nous étudions l'incorporation dans les lipides hépatiques et sériques de rats non sevrés, de rats recevant une ration commerciale et de rats recevant une ration semi-synthétique sans addition de graisse, avec 15% de beurre ou avec 15% d'huile de maïs. L'incorporation dans le cholestérol hépatique est plus élevée chez les rats recevant la ration commerciale que chez ceux recevant les rations semi-synthétiques et elle est stimulée par l'addition d'huile de maïs à chacune des diètes. Dans les différents groupes alimentaires, l'incorporation dans les acides gras n'est pas parallèle à l'incorporation dans le cholestérol. Dans les sériques sanguins, le marquage tend à ressembler à celui des lipides hépatiques. Chez les rats non sevrés, l'incorporation est faible et dans le cholestérol et dans les acides gras. En général, ces résultats correspondent aux données antérieures d'études *in vitro*.

Introduction

Earlier investigations in our laboratory were concerned with effects of diet on incorporation of acetate into cholesterol and fatty acids by rat liver slices *in vitro*, and it was shown that incorporation into cholesterol was much higher in rats on commercial diets than in rats on semisynthetic diets (1, 2). Addition of unsaturated fats to these diets was found to stimulate incorporation more than addition of saturated fats, in agreement with results from other laboratories. These dietary effects seemed to be fairly specific for acetate incorporation into cholesterol since incorporation into fatty acids, measured in the same experiments, did not show similar differences. Other experiments showed that liver slices from suckling rats incorporated very little acetate into either cholesterol or fatty acids (3). From these studies, it appeared that there might be an inverse relationship between the level of plasma cholesterol and incorporation of acetate into liver cholesterol, since suckling rats were found to have significantly elevated plasma cholesterol levels and it was known that rats on semisynthetic diets tend to have higher serum cholesterol levels than rats on commercial diets (2).

Our earlier studies were concerned mainly with *in vitro* experiments. The present communication deals with an extension of this work to *in vivo* studies, in which 1-^{14}C-acetate was injected intravenously and its incorporation into liver and serum lipids was determined in rats under various dietary conditions.

Methods

The rats used for these experiments belonged to the Sprague–Dawley strain. They were either obtained from Sprague–Dawley Inc., Madison, Wisconsin, or were raised in our own laboratory. Male rats only were used for the studies with experimental diets, but the group of suckling rats contained animals of both sexes. The following diets were used: Commercial diet (Master Fox Breeder Starter Ration, Master Feeds, Toronto, Ontario); commercial diet extracted with ethyl ether to remove most of the lipid; and fat-free semisynthetic diet based on casein and dextrose (1, 2). Butter or corn oil was added to the semisynthetic

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and ether-extracted commercial diets at a level of 15% by weight. The feeding period was 3 weeks in each case.

On the day of the experiment, about 10 a.m., each rat was given 10 μCi/100 g body weight of 1-^{14}C-acetate (specific activity 40 mCi/mmol) in 0.1–0.12 ml of physiological saline, by tail vein injection. One hour later, the animals were anesthetized with ether, a sample of blood was taken by heart puncture, and the liver was removed and homogenized with chloroform–methanol, 2:1 (4 ml/g of fresh liver) in a Virtis homogenizer. The supernatant was filtered, and the residue was reextracted with another similar amount of chloroform–methanol. The extracts from all livers in each group were pooled and washed with water as described by Folch *et al.* (4). They were dried over Na_2SO_4, and the solvent was removed *in vacuo*.

The blood was left to coagulate at room temperature, and the serum was separated by centrifugation. The serum from all rats in each group was pooled and extracted twice with chloroform–methanol, 2:1 (6 ml/ml of serum), and the extracts were washed and dried in the same way as the liver extracts.

Analysis of Liver Lipids

From 50 to 100 mg of total lipid from each experimental group were separated into neutral lipids and phospholipids by chromatography on a 5 g column (1 × 16 cm) of acid-treated Florisil (5). Neutral lipids were eluted with 100 ml of chloroform, and phospholipids with 100 ml of methanol. For determination of total cholesterol and total fatty acids in neutral lipids an aliquot was hydrolyzed in 8 ml of ethanol and 4 ml of 33% aqueous KOH at 45°C for 1 h with occasional shaking. The reaction mixture was acidified with concentrated HCl and extracted with Skellysolve B or hexane. The extracted fatty acids and cholesterol were then separated quantitatively by chromatography on a 12 g column (1.2 × 20 cm) of acid-treated Florisil, eluting with hexane–ether (5). Although nonsaponifiable lipids such as cholesterol are often separated from fatty acids by extracting the alkaline hydrolysate with petroleum ether and then reextracting the acidified hydrolysate to obtain the fatty acids, this method proved unsatisfactory because some fatty acids were always extracted with the nonsaponifiable fraction. The phospholipids were hydrolyzed in the same way and their total fatty acids determined.

Incorporation of 1-^{14}C-acetate into the lipids, separated as described above, was determined by counting aliquots in a Nuclear-Chicago model D-47 gas-flow counter, with Micromil window (counting efficiency 30%). In addition, the distribution of radioactivity in different lipid classes was determined by separating them on thin-layer chromatograms and scanning the plates with an Actigraph II model C1003 (Nuclear-Chicago). For this purpose, 3–5 mg aliquots of total liver lipids were spotted on plates of silica gel H (50 × 500 mm) and developed with heptane–ether–acetic acid–methanol (85:15:2:3). In this system, phospholipids remain at the origin, and diglycerides run close to cholesterol, but other major neutral lipid classes are cleanly separated (6). After the plates had been scanned for radioactivity, the positions of the different lipid bands were determined by spraying with 50% sulfuric acid and charring.

Serum Lipids

An aliquot of the serum lipids was separated by column chromatography into neutral and phospholipids as described above for liver lipids. The remainder was used for thin-layer radiochromatography. The quantities and radioactivities of the separated neutral and phospholipid fractions were determined as before, and they were then recombined and hydrolyzed for determination of total cholesterol and total fatty acids.

Results

Data on the composition and radioactivity of liver lipids 1 h after intravenous injection of 1-^{14}C-acetate into rats on the different diets are presented in Table 1. The results given for the different dietary groups are typical of those obtained when the experiments were repeated on at least one or two other occasions.

The total amount of liver lipid was similar for most diets but was higher in corn oil fed rats, due to an increase in neutral lipids. In most cases the neutral lipids contained more radioactivity than the phospholipids and their specific activity was often considerably higher.

Rats on commercial diets incorporated more 1-^{14}C-acetate into cholesterol than rats on semisynthetic diets. Addition of corn oil to the diets increased the amount of cholesterol in the liver and stimulated incorporation of the labeled acetate into cholesterol. In the case of the semisynthetic diets, this represented an increase in both the absolute and relative specific activity. On the other hand, the specific activity of neutral lipid fatty acids relative to phospholipid fatty acids decreased from a ratio of approximately 4:1 on low fat diets to about 1:1 on corn oil diets. This low ratio was largely due to the increased amounts of neutral lipid fatty acids in corn oil fed rats. Suckling rats showed low incorporation, particularly with respect to the cholesterol and neutral lipid fatty acid fractions.

It can be seen from Fig. 1 that the radioactivity in the neutral lipids was present mainly in the triglyceride and free cholesterol fractions. This figure also shows the higher activity of cholesterol relative to triglycerides and phospholipids in rats on commercial diets and the increased incorporation into cholesterol when corn oil was added to the semisynthetic diet. The tracings for the butter-containing diets were rather similar to those obtained for the corresponding low fat diets.

Serum Lipids

The values for composition and radioactivity of serum lipids 1 h after intravenous injection

TABLE 1. Composition and radioactivity of liver lipids of rats fed different diets and injected intravenously with 1-^{14}C-acetate

Diet	No. of rats	Avg. body wt. (g)	Avg. liver wt. (g)	Lipid composition (mg/g liver)						Incorporation of 1-^{14}C-acetate (c.p.m./g liver)						Specific activity relative to PL					
				Total lipid	NL	NL-FA	C	PL	PL-FA	Total lipid	NL	NL-FA	C	PL	PL-FA	Total lipid	NL	NL-FA	C	PL	PL-FA
Suckling	22	30	1.05	46.5	10.8	7.5	2.4	29.7	22.8	2820	930	370	150	1915	1235	1.1	1.3	0.8	1.0	1.0	0.8
Semisynthetic	4	210	8.1	42.6	10.4	7.3	3.1	31.6	28.1	9970	4720	3380	280	3350	2780	2.0	4.3	4.4	1.2	1.0	0.9
Plus 15% butter	6	198	8.0	43.9	11.7	8.1	2.2	31.2	23.6	4650	2000	1520	150	2430	2340	1.4	2.3	2.4	0.9	1.0	1.3
Plus 15% corn oil	4	215	7.3	61.5	24.6	17.5	3.7	33.4	24.9	3993	2125	950	900	1690	1485	1.3	1.7	1.1	4.8	1.0	1.2
Ether-extracted commercial	4	205	7.9	40.2	9.5	7.2	2.0	28.4	21.7	4000	2690	1260	690	1260	1000	2.3	6.4	4.0	8.7	1.0	1.0
Plus 15% butter	6	213	8.1	38.1	9.0	6.5	1.9	29.1	20.8	5150	2900	1620	690	2150	2000	1.8	4.5	3.4	7.0	1.0	1.3
Plus 15% corn oil	4	205	8.0	63.2	32.5	24.0	3.9	28.7	21.8	5950	3360	1520	1375	2330	2030	1.1	1.2	0.8	5.5	1.0	1.1
Commercial	6	210	8.2	36.2	7.6	5.1	2.1	28.6	21.4	10700	5670	3370	1730	5400	3190	1.6	4.0	3.5	3.4	1.0	0.8

NOTE: NL, neutral lipids; C, cholesterol; PL, phospholipids; FA, fatty acids.

TABLE 2. Composition and radioactivity of serum lipids of rats fed different diets and injected intravenously with 1-^{14}C-acetate

Diet	Lipid composition (mg/ml serum)					Incorporation of 1-^{14}C-acetate (c.p.m./ml serum)					Specific activity relative to PL				
	Total lipid	NL	C	PL	TFA	Total lipid	NL	C	PL	TFA	Total lipid	NL	C	PL	TFA
Suckling	5.8	3.7	1.38	2.1	3.2	355	200	40	50	225	2.4	3.1	1.1	1.0	2.8
Semisynthetic	3.7	2.1	0.52	1.5	2.0	825	670	40	140	600	2.4	3.4	0.7	1.0	3.2
Plus 15% butter	4.4	2.7	0.99	1.6	2.1	685	400	50	135	380	1.9	1.8	0.5	1.0	2.2
Plus 15% corn oil	3.7	2.1	0.89	1.5	1.8	325	260	130	60	135	2.1	3.0	3.5	1.0	2.6
Ether-extracted commercial	3.9	2.2	0.76	1.6	2.0	570	400	60	130	345	1.9	2.4	1.0	1.0	2.3
Plus 15% butter	4.7	3.0	0.83	1.6	2.3	1390	1210	350	140	660	3.4	4.5	4.7	1.0	3.4
Plus 15% corn oil	3.9	2.2	1.05	1.6	1.9	530	450	370	80	150	2.6	4.0	4.8	1.0	1.5
Commercial	4.2	2.5	0.87	1.8	1.9	1715	1360	150	345	855	2.1	2.8	0.9	1.0	2.3

NOTE: These results are from the same experiments as those in Table 1. NL, neutral lipids; C, cholesterol; PL, phospholipids; TFA, total fatty acids.

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of the labeled acetate are shown in Table 2. The different experimental diets did not have any marked effects on serum lipid levels, but neutral lipids, phospholipids, and cholesterol were all higher in suckling rats. With regard to radioactivity, the overall pattern was somewhat similar to that seen in the liver lipids. The specific activity of the neutral lipids was higher than that of the phospholipids in all cases; and, since the amount of neutral lipid relative to phospholipid is much higher in serum than in liver, the neutral lipids of serum contained an even higher proportion of the radioactivity than those of liver.

Rats on commercial diet incorporated more radioactivity into serum cholesterol than those on semisynthetic diets; and, as with the liver lipids, addition of corn oil enhanced the incorporation into cholesterol. However, unlike the results for liver, the specific activity of serum cholesterol was quite low in the rats on low fat commercial diet as well as in those on low fat semisynthetic diet. This can also be seen in the charts depicted in Fig. 2. In addition, the charts illustrate the high levels of radioactivity in neutral lipids relative to phospholipids noted above. Suckling rats showed relatively low incorporation into cholesterol and fatty acids, as in the case of the liver lipids.

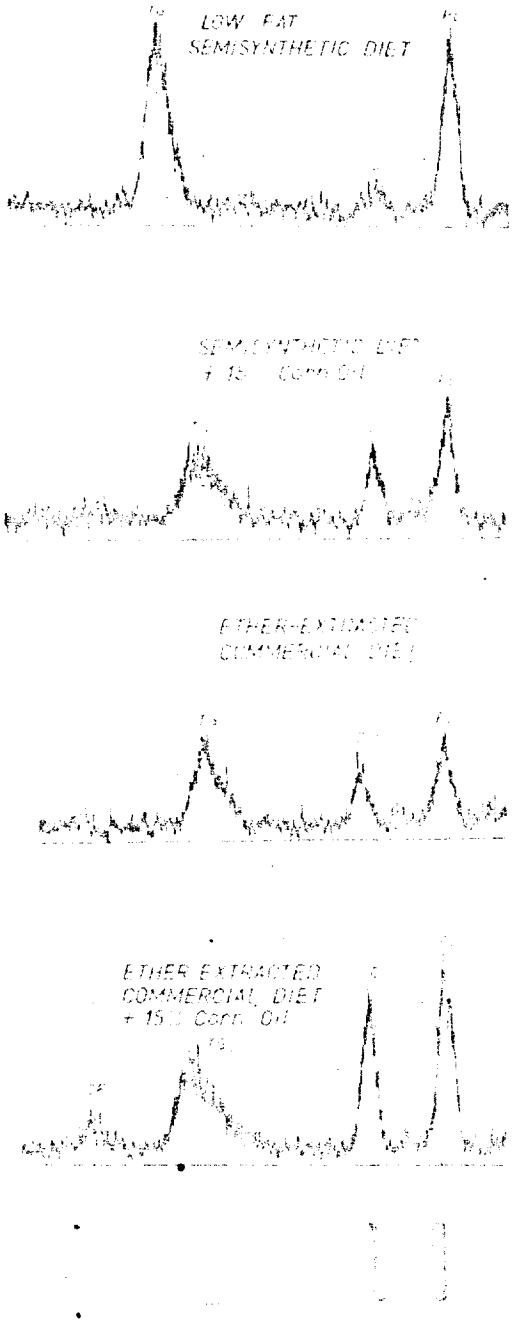

FIG. 1. Distribution of radioactivity in liver lipids of rats on different diets 1 h after intravenous injection of acetate-1-^{14}C. The lipids were separated by thin-layer chromatography and the chromatograms were scanned for radioactivity and then charred to show the locations of different lipid classes. A single chromatogram is shown at the bottom. CE, cholesterol esters; TG, triglycerides; C, cholesterol; PL, phospholipids.

Discussion

The results of these *in vivo* experiments confirm our earlier findings *in vitro* (1–3) that rats on commercial diets incorporate more acetate-1-^{14}C into liver cholesterol than rats on semisynthetic diets or suckling rats. The results also show that diets containing corn oil give higher incorporation into cholesterol than low fat diets or diets containing butter, in agreement with our previous findings. In general, the pattern of incorporation into serum cholesterol tended to parallel that for liver cholesterol (Tables 1 and 2).

As in the earlier *in vitro* experiments, incorporation into fatty acids did not follow the same trend as incorporation into cholesterol in

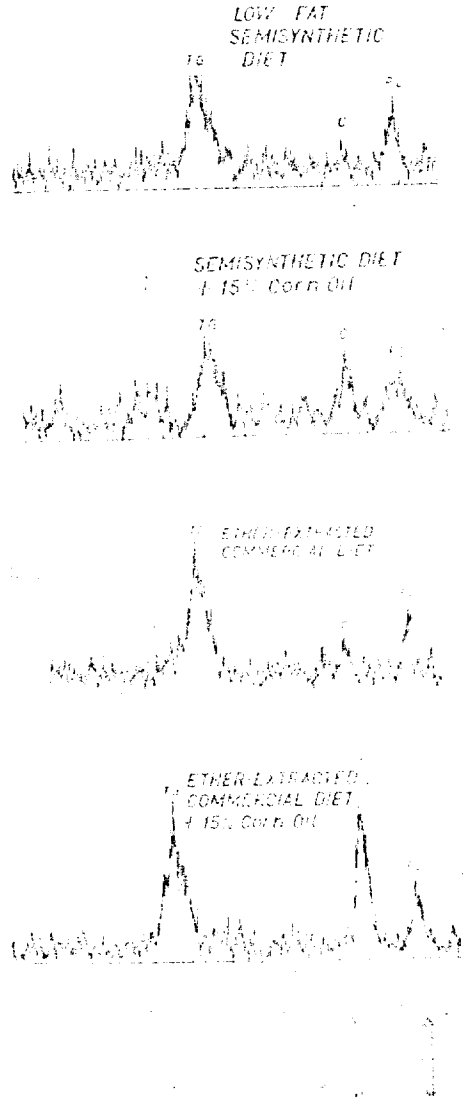

FIG. 2. Distribution of radioactivity in serum lipids of rats on different diets 1 h after intravenous injection of acetate-1-^{14}C. For procedures and abbreviations see Fig. 1.

the different dietary groups. Suckling rats showed low incorporation into fatty acids as well as cholesterol, and this also agrees with our previous findings.

Dupont (7) studied the *in vivo* incorporation of 1-^{14}C-acetate in rats on semisynthetic diet, and found that incorporation into liver and serum cholesterol was stimulated by a high level of corn oil in the diet. She also reported that incorporation of acetate into liver fatty acids was depressed when the diet contained either beef tallow or corn oil. In our experiments, addition of either butter or corn oil to the semisynthetic diet decreased incorporation into fatty acids, but this did not occur when the fats were added to commercial diet (Table 1). It should be kept in mind, however, that all of our rats were killed 1 h after injection of the labeled acetate and the picture might be different at other time intervals.

The reason for the higher incorporation of acetate into cholesterol on commercial diets is not known but other studies in our laboratory have shown that rats on commercial diets convert 26-^{14}C-cholesterol to respiratory $^{14}CO_2$ at a more rapid rate than rats on semisynthetic diets (8), and the increased rate of degradation may act as a stimulus to increased biosynthesis. In those experiments there was no indication that dietary corn oil enhanced the rate of catabolism of cholesterol but there is some evidence that excretion of fecal sterols is increased by feeding corn oil (9–10).

Dupont (11, 12) has considered possible mechanisms by which dietary corn oil might stimulate cholesterol biosynthesis. Linoleic acid is the most abundant fatty acid in corn oil, and serum cholesteryl esters contain high proportions of linoleic acid and its metabolic product, arachidonic acid. Feeding corn oil might increase the turnover rate of the fatty acid moiety of cholesteryl esters and this in turn might lead to increased catabolism or excretion of cholesterol (11). The acceleration of acetate incorporation into cholesterol by dietary fat does not appear to be due to formation of acetyl-CoA from the fatty acids and incorporation of that acetate into cholesterol (12).

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SUBSTITUTION OF VINEGAR FOR LACTIC ACID AS BACTERICIDAL AGENT IN INFANT MILK MIXTURES

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BUTTERMILK was introduced as an infant food in Sweden in 1776, in Holland in 1865, in Germany in 1902, in France in 1907, and in the United States in 1913,* because it was found that infants thrived better on it than on ordinary sweet milk.† Other sour-milk mixtures soon followed. Clark and Faber attributed the value of the acid to an improvement in the digestibility of the milk.[4] They showed that the buffer value of cows' milk is considerably more than that of human milk, that it neutralizes a larger amount of the hydrochloric acid of the stomach, and thus may interfere with the activity of the gastric enzymes through a reduction of the gastric acidity below the optimal hydrogen-ion concentration zone of pH 3.5 to 5.0. The addition of an acid to cows' milk counteracted its excess buffer salts and made its digestibility more nearly that of human milk. Because of this and similar work,‡ many types of soured milk became popular for infant feeding in the United States. Lemon juice, citric acid, hydrochloric acid, and vinegar (acetic acid) have been used, but lactic acid milk has the widest use at present.‡

While it may be true that soured milk is more digestible than sweet milk[4] and that soft curds are important,§ the most likely reason that infants thrive better on soured milk and have fewer digestive upsets is the suggestion in 1927, by Marriott,[8] and the demonstration in 1935, by Rothey,[12] that milk containing 0.66% or more of lactic acid U. S. P. was bactericidal for dysentery, typhoid, and other organisms. Taylor and Roberts, in 1943, found that lactic acid milk could be left in open dishes at room temperature for three days before becoming contaminated by molds and for five days before bacteria appeared.[20] This bactericidal action has been clinically demonstrated at Duke Hospital for the past 20 years by the rarity of diarrhea and

From the Department of Pediatrics, Duke University School of Medicine and Duke Hospital.

* References 1-3.

† References 5-11.

‡ References 1 and 12-14.

§ References 3, 15-18.

dysentery in infants fed lactic acid milk.‖ The main disadvantages of using lactic acid are, first, that it is a poison if too strong a solution is used (30 cc. [1 fluidounce] of 85% lactic acid can be fatal),¶ and, second, that it is expensive.

Apple cider vinegar, which is cheap, nonpoisonous, and can be obtained at any grocery, is suggested in place of lactic acid. Apple cider vinegar contains 5 to 6% acetic acid, with variable traces of other harmless ingredients. Dunham, in 1925, fed various mixtures of apple vinegar and cows' milk to 40 infants over a period of one to six months.[21] The most satisfactory mixture was 30 cc. (1 oz.) of vinegar to 450 cc. (15 fluidounces) of cows' milk, or a final dilution of 6.66% of vinegar. The acetic acid content of this mixture is 0.33 to 0.40%. The general appearance, contentment, nutrition, tissue turgor, musculature, and rate of growth of these vinegar-milk-fed babies paralleled those of the infants fed lactic and citric acid mixtures. Their digestion was good; vomiting, flatulence, and refusal of the mixture were not encountered. The substitution of vinegar for the other acids did not noticeably change the clinical signs. The preparation of the vinegar-milk is similar to that of other acidified milk mixtures[22] (Appendix).

TABLE 1.—*Bactericidal Effect of Apple Cider Vinegar (Five to Six Per Cent Acetic Acid) on the Growth of Organisms Inoculated in an Infant Feeding Milk Mixture*

Milk Mixture, Cc.	Vinegar		Organisms*				
	Amount, Cc.	Concentr., %†	S. dys.	S. parad.	S. typh.	S. paat.	Esch. coli
10.0	0 (control)	0 (0)	0	0	0	0	0
9.9	0.1	1 (0.05-0.06)	0	0	0	0	0
9.8	0.2	2 (0.1-0.12)	0	0	0	0	0
9.7	0.3	3 (0.15-0.18)	0	0	0	0	0
9.6	0.4	4 (0.2-0.24)	+	0	+	+	0
9.5	0.5	5 (0.25-0.3)	+	+	+	+	+

* S. dys. indicates Shigella dysenteriae; S. parad., Shigella paradysenteriae; S. typh., Salmonella typhosa; S. paat., Salmonella paratyphi B; Esch. coli., Escherichia coli; + indicates complete bactericidal effect, i. e., no viable bacteria.

† The first figures indicate the percentages of vinegar (5-6% acetic acid) and those in parentheses indicate the concentration of acetic acid.

Higher percentages of vinegar may exert an alkaline diuretic effect, but Sollmann fed massive doses to rats, equivalent in man to 240 cc. (8 fluidounces) of vinegar, daily for several months without any adverse effects. The addition of vinegar to the mash fed to young calves has reduced the incidence of scours (diarrhea).[26] A teaspoonful of apple cider vinegar (5 cc.) in a glass of water every four hours also is said to cure diarrhea in adults.[27]

The bactericidal effect of an increased acidity is well established. However, acetic and certain other organic acids appear to have a bactericidal effect in excess of that which could possibly be due to the pH alone. Kirby, in 1936, found that acetic acid or vinegar inhibited bread molds. At the same pH, greater inhibition was obtained with acetic acid. Collet, in 1919, found that in solutions of equal pH butyric and acetic acids were more bactericidal than hydrochloric and other strong acids. Levine concluded that organisms were inhibited or destroyed at a lower acidity when acetic acid was employed than when lactic or hydrochloric acids were

‖ References 1 and 21.

¶ References 22 and 23 and Hartmann, A. F.: Personal communication to the authors.

used. Tab[illegible]g, in 1927, showed that alkali salts alone of several organic acids had no marked bactericidal effect. Vinegar has been used effectively for generations to preserve sauerkraut and pickles.

Bactericidal Effect of Apple Cider Vinegar (Acetic Acid) and Lactic Acid.—As indicated in Tables 1 and 2, 9.5 to 10 cc. of an infant-feeding milk mixture, comparable to the lactic acid mixtures used in our clinic (510 cc. [17 fluidounces] of water, 360 cc. [13 fluidounces] of evaporated milk, and 30 cc. [2 tablespoonfuls] of sugar [Appendix]) were transferred to two series of sterile test tubes to which 0 to 0.5 cc. of apple cider vinegar and lactic acid had been added, respectively, in amounts to produce total volumes of 10 cc. and acid concentrations of 0 (control) to 5%. Twenty-four-hour broth cultures of Shigella dysenteriae, Shigella paradysenteriae, Salmonella typhosa, Salmonella paratyphi, and Escherichia coli, in 0.1 cc. amounts, were added to each series of these tubes of acid milk, which were then

Table 2.—Bactericidal Effect of U. S. P. Lactic Acid (Eighty-Five Per Cent Lactic Acid) on the Growth of Organisms Inoculated in an Infant Feeding Milk Mixture

Milk Mixture, Cc.	Lactic Acid Amount, Cc.	Lactic Acid Concentr. % †	Organism * S. dys.	S. parad.	S. typh.	S. parat.	Esch. coli
10.0	0 (control)	.....	0	0	0	0	0
9.5	0.5	1 (0.85)	+	+	+	+	+
9.8	0.2	2 (1.7)	+	+	+	+	+
9.7	0.3	3 (2.55)	+	+	+	+	+
9.6	0.4	4 (3.4)	+	+	+	+	+
9.5	0.5	5 (4.25)	+	+	+	+	+

* S. dys., indicates Shigella dysenteriae; S. parad., Shigella paradysenteriae; S. typh., Salmonella typhosa; S. parat., Salmonella paratyphi B; Esch. coli., Escherichia coli; + indicates complete bactericidal effect, i. e., no viable bacteria.

† The first figures indicate the percentage of U. S. P. lactic acid (85%), and those in parentheses indicate the actual concentration of lactic acid.

incubated at 37.5 C. for 24 hours, streaked on eosin-methylene blue plates, and incubated for 48 hours. The presence or absence of growth on these plates (viable bacteria) is recorded in Tables 1 and 2.

CONCLUSIONS

1. Apple cider vinegar in a concentration of 4%, or 1 part of vinegar to 25 parts of an infant feeding milk mixture (0.2 to 0.24% acetic acid), prevents the growth of pathogenic bacteria.

2. Vinegar is a safe addition to milk to prevent pathogenic contamination.

3. Unlike lactic acid, vinegar is harmless in the proportions recommended.

4. Vinegar is easily obtainable and is cheap.

5. Vinegar milk is easily prepared, is palatable, and forms a soft smooth curd which flows easily through a nipple.

6. Infants thrive on vinegar milk (1 part of vinegar to 15 parts of cows' milk, i. e., 5.66% vinegar or 0.33 to 0.4% acetic acid).[24]

7. Vinegar milk retains all of the virtues of lactic acid milk,[1] but has none of its disadvantages and, in addition, is cheaper and easier to obtain.

APPENDIX

Home Instructions for the Preparation of Vinegar Evaporated Milk

Ingredients.—A 13-fluidounce can of unsweetened evaporated whole milk, 17 fluidounces water, 2 tablespoonfuls of sugar, and 3 tablespoonfuls of apple cider vinegar. The caloric content of this mixture is 22 Cal. per fluidounce. It can be used for normal infants from birth to 8 months of age. For older children, omit the sugar and reduce the calories to 20 per fluidounce.

Preparation.—Scald with boiling water a clean quart jar or milk bottle and an empty clean 13-fluidounce evaporated milk can. Fill this measuring can with water and pour it into the quart jar or milk bottle. Add 2 level standard tablespoonfuls of ordinary table sugar and 3 standard tablespoonfuls of ordinary apple cider vinegar to this water. As soon as the sugar has dissolved, cool this sugar and vinegar solution. Then open a 13-fluidounce can of unsweetened evaporated whole milk and pour the milk slowly into the quart jar or milk bottle containing the cold sugar and vinegar solution with continuous gentle shaking. Last, fill the quart jar or bottle of evaporated milk, water, sugar, and vinegar nearly to the top with cold water and reshake to make the mixture smooth. The mixture should not be boiled or pasteurized, as it will curdle. Refrigeration is unnecessary.[5b]

If the water supply is bad or is well or pump water, the water should be boiled and cooled before using. A standard level table-spoon contains 12 gm. of sugar or 15 cc. of vinegar. Those obtained from several farms contained 7 to 15 gm. of sugar or 9 to 19 cc. of vinegar.# Ordinary table sugar is as good as Karo syrup or any other sugar. Chilling the sugar and vinegar solution facilitates mixing and makes a better curd. Do not pour the solution into the milk.

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THE EFFECT OF INSULIN ON THE UTILIZATION OF [U-^{14}C]GLUCOSE AND [1-^{14}C]ACETATE BY GOAT ADIPOSE TISSUE *IN VITRO*

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SUMMARY

No change in the rate of $^{14}CO_2$ production from [U-^{14}C]glucose by the adipose tissue of goats was found *in vitro*, even in the presence of high concentrations of insulin (1 and 10 m-u./ml.) when glucose was the only substrate in the medium. However, it was demonstrated that in the presence of acetate as little as 10 μu. insulin/ml. exerted a marked effect on glucose oxidation. The most significant effect of insulin was that on the rate of [1-^{14}C]acetate incorporation into fatty acids in the presence of glucose. These findings support the suggestion that the significance of insulin in ruminants is best demonstrated by its effects on the rate of utilization of acetate in the presence of glucose by adipose tissue.

INTRODUCTION

Recent reports of Manns, Boda & Wiles (1967) and Horino, Machlin, Hertelendy & Kipnis (1968) have demonstrated that the levels of insulin in the blood of ruminants vary within similar limits as in non-ruminants. However, it is well known that the decline of blood glucose after insulin administration is much less in ruminants. The weak effect of insulin on the blood glucose level could be related to a relatively high level of plasma acetate wh[illegible] component of the blood of ruminants. Most of the acetate is of exogen[illegible] and derives from ruminal fermentation of carbohydrates. It seems that the higher levels of blood acetate tend to increase the intramitochondrial concentration of acetyl CoA and that the decrease of the CoA:acetyl-CoA ratio inhibits the oxidation of pyruvate to acetyl-CoA (Garland & Randle, 1964). Moreover, citrate which passes across the mitochondrial membrane into the cytoplasm cannot be cleaved into oxaloacetate and acetyl-CoA because of the absence of ATP citrate lyase (Hardwick, 1965). This makes the formation of fatty acids from glucose in ruminants difficult. However, insulin may induce: (1) An increased uptake of glucose by adipose tissue. This would result in an increased availability of α-glycerolphosphate, an increased rate of esterification and a decrease of fatty acids release. (2) An increased metabolism of glucose by way of the pentose cycle. This would result in an increased availability of $NADPH_2$ and thus in an increased incorporation of acetate into fatty acids.

According to this concept, recording blood glucose changes after insulin administration has little meaning for the investigation of the effect of insulin in ruminants and it is more advisable to study the utilization of glucose and acetate by adipose tissue. To confirm this suggestion the effect of insulin on glucose and acetate utilization by adipose tissue *in vitro* has been studied in the goat.

MATERIALS AND METHODS

The goats used were crossbred males, 3–4 months old. The animals were bled and samples of adipose tissue were obtained from the greater omentum. The tissue was kept at 38–40° in 0·9% NaCl solution before use. The incubation medium was Krebs–Ringer bicarbonate buffer containing 2% bovine serum albumin (Armour fraction V) and half of the usual concentration of Ca^{2+}. To the medium was added [U-^{14}C]glucose, 50 mg./100 ml. (1·0 μc/flask) and [1-^{14}C]acetate, 5 mM (1·0 μc/flask). The tissue was incubated at 38° in an atmosphere of O_2 and CO_2 (95:5) for 3 hr. in polyethylene flasks to prevent the adsorption of insulin to the walls. At the end of the incubation the liberated CO_2 was trapped in 50% monoethanolamine in ethanol. The monoethanolamine was transferred to a scintillation vial and 15 ml. of toluene scintillation solution were added.

For the radioactivity measurement of whole lipids the tissue was rinsed several times in 0·9% NaCl solution, transferred to a tube containing 15 ml. chloroform: methanol (2:1, v/v), and extracted for 6 hr. with continuous shaking (Leveille, 1967). This procedure has been shown by gravimetric analysis to remove 98–99% of the lipids. The lipid extract was washed twice according to Folch, Lees & Stanley (1957) to remove nonlipid radioactivity.

The radioactivity in fatty acids was measured after the evaporation of the aliquots of the chloroform: methanol extracts, saponification and extraction with chloroform. Aliquots of both lipid and fatty acids extracts were transferred to a scintillation vial. The solvent was evaporated and dissolved in 10 ml. toluene scintillation solution. The difference between both measurements was considered as radioactivity due to glycerol.

All radioactivity determinations were performed in a Mark I Nuclear–Chicago liquid scintillation spectrometer.

Results are expressed as counts/min./mg. wet adipose tissue.

RESULTS AND DISCUSSION

No changes in the rate of $^{14}CO_2$ production from [U-^{14}C]glucose was observed in goat adipose tissue even in the presence of high concentrations of insulin (1 and 10 m-u./ml.) when glucose was the only substrate in the medium. However, a marked effect of insulin on glucose oxidation was found in the presence of acetate (Fig. 1).

The effect of insulin at concentrations from 10 to 1000 μu./ml. on the incorporation of radioactive carbon from [U-^{14}C]glucose into CO_2 and glycerol is shown in Fig. 2.

The curve of $^{14}CO_2$ production from glucose has a sigmoid shape and the rate of the $^{14}CO_2$ formation was already increased at an insulin concentration of 10 μu./ml. In contrast, the changes in the conversion of [U-^{14}C]glucose into glycerol were sc

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and were found only at high concentrations of insulin. This is not surprising. The requirements for glucose for the formation of glyceride glycerol are relatively low as compared with the amounts of glucose which were to be broken down by way of the

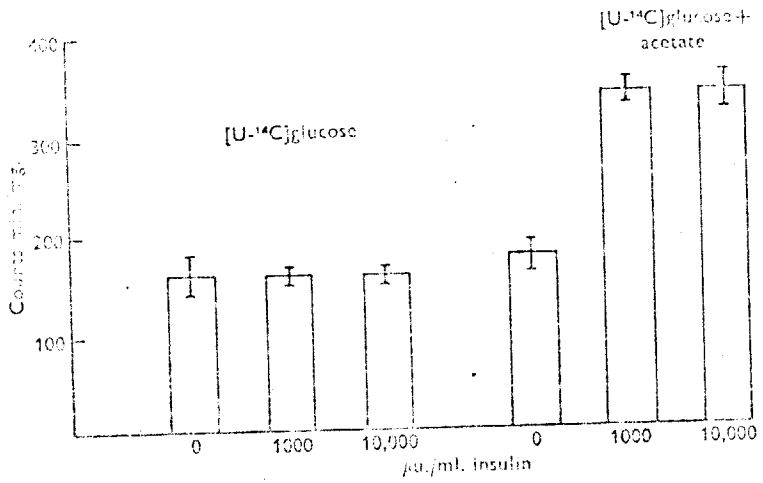

Fig. 1. Effect of insulin and acetate on the incorporation of [U-^{14}C]glucose into CO_2 by adipose tissue of goat *in vitro* (expressed as counts/min./mg. wet tissue). Substrate concentration in medium: glucose, 50 mg./100 ml.; acetate, 5 mM. Means ± S.E. from five tissue samples.

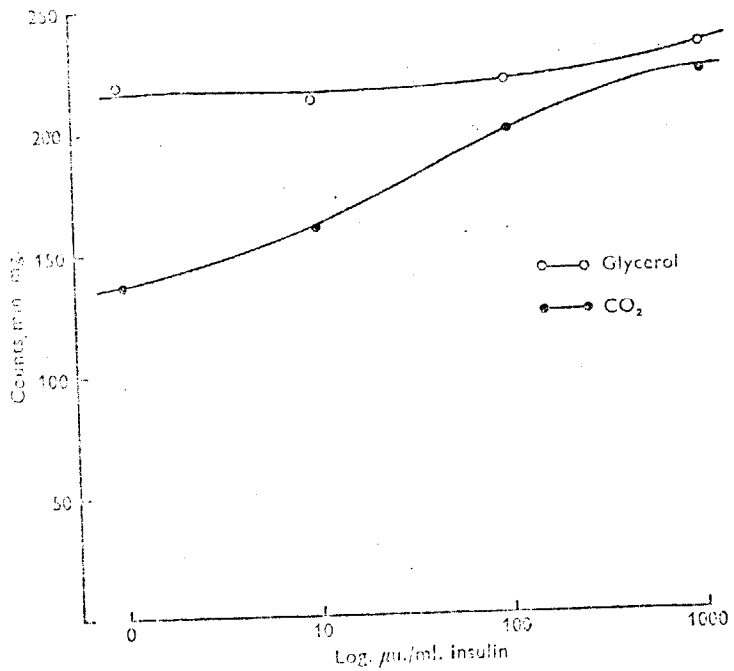

Fig. 2. Effect of increasing the concentration of insulin in the medium on the conversion of [U-^{14}C]glucose into CO_2 and glycerol in the presence of acetate by goat adipose tissue *in vitro*. (Counts/min./mg. wet tissue). Substrate concentration in medium: glucose, 50 mg./100 ml.; acetate, 5 mM. The points represent mean values from six tissue samples.

pentose cycle to form adequate amounts of $NADPH_2$ for the incorporation of acetate into fatty acids. However, the amount of radioactivity found in the glycerol fraction of the whole lipids was higher than that in the liberated CO_2. In this context it should be noted that α-glycerophosphate is required not only for the esterification of fatty acids formed from added acetate, but that there are also considerable requirements for α-glycerophosphate for the enhanced re-esterification of long-chain fatty acids released from adipose tissue by lipolysis. These re-esterification processes of long-chain fatty acids make no further demands on $NADPH_2$.

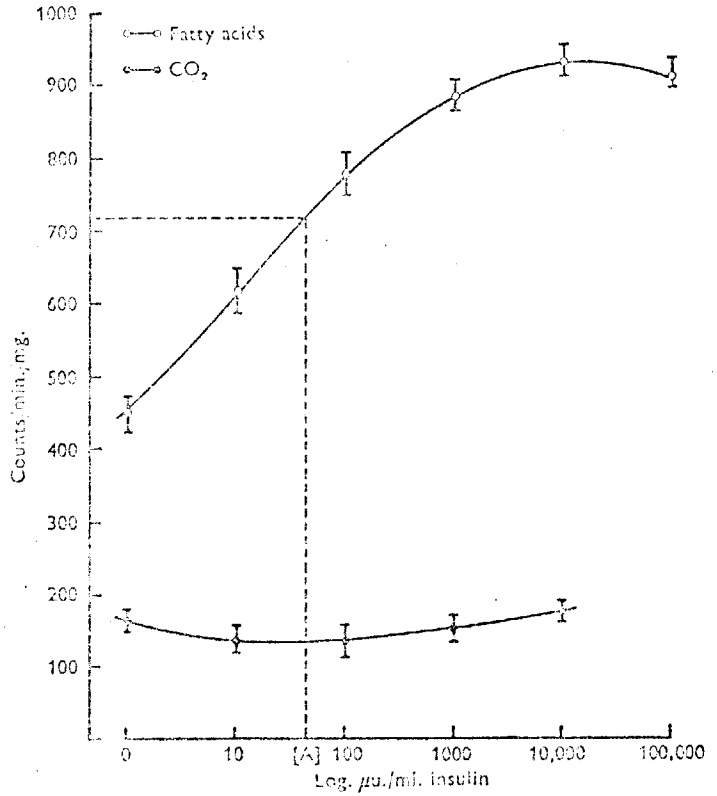

Fig. 3. Effect of increasing the concentration of insulin in the medium on the incorporation of [1-^{14}C]acetate into fatty acids and CO_2 in the presence of glucose by goat adipose tissue *in vitro* (count/min./mg. wet tissue). Substrate concentration in medium: glucose, 50 mg./100 ml.; acetate, 5 mM. Means ± S.E. from six tissue samples. [A] = concentration of insulin (about 40 μu./ml.) which gave half of the maximal effect.

The incorporation of glucose into fatty acids was too low (1·3–2·2 counts/min./mg. wet adipose tissue) to be plotted in Fig. 2.

The most significant effect of insulin was that on the rate of [1-^{14}C]acetate incorporation into fatty acids in the presence of glucose (Fig. 3). The curve of radioactivity of fatty acids has again a typical sigmoid shape; the maximum was reached at an insulin concentration of 10 m-u./ml., but at 100 m-u./ml. the curve already tends to decline. According to the receptor theory this decline at the high concentrations of

insulin might be interpreted as auto-inhibition (Weeke, 1967). It can be derived from the curve that the concentration of insulin causing half of the maximal effect is about 40 μu./ml.

The incorporation of [1-^{14}C]acetate into CO_2 was not markedly affected. Only at lower concentrations of insulin (10–100 μu./ml.) could a slight depression of $^{14}CO_2$ production be detected.

It can be concluded that our results fully support the suggestion that the significance of insulin in ruminants could be demonstrated best in its effects on the rate of utilization of acetate in the presence of glucose by adipose tissue.

There are numerous experiments in dairy cows on the effect of high concentrate and restricted roughage diets on milk production. These diets increase the ratio of propionate to acetate in the rumen and tend to lower milk fat. Recently Manns *et al.* (1967) and Horino *et al.* (1968) have shown that propionate exerts a marked effect on the secretion of insulin; therefore the observed depression of milk fat could be explained by an increased rate of insulin secretion which, in turn, would account for increased utilization of acetate and reesterification in the adipose tissue and a decreased availability of acetate and long-chain fatty acids in the mammary gland.

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Absorption of Voltile Fatty Acids and Their Salts in Sheep Abomasum

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As a result of bacterial processes occurring in the rumen of ruminants, the nutrient substances present in feed are broken down to form a number of products, among which special significance is attributed to organic acids with a short carbon chain, namely volatile fatty acids (VFA).

In recent years, Soviet and foreign scientists have clarified a number of characteristic features in the absorption of VFA from certain sections of the digestive tract. It was shown that the main fermentation acids, namely acetic, propionic and butyric acids are actively absorbed at the site where they are formed, i.e. the rumen and omasum (4,9,11,16). However, not all VFA are absorbed in the rumen, and a considerable fraction of these acids can be transferred to lower sections of the digestive tract, namely into the abomasum and intestine (5,17). In ruminants, the continuously absorbed low-molecular acids are utilized to supply energy requirements of the organism and in the synthesis of some organic compounds, such as glucose, milk, fat, etc. (10,12,18).

So far, little is known about the way in which VFA and their salts are absorbed in the abomasum, or true stomach of ruminants. The task of the present investigation was to study the absorption of VFA and their salts in the abomasum of sheep.

Method

Tests on the study of the absorption of VFA and their salts were carried out on 3 female sheep of the Carpathian mountain species, with isolated abomasums prepared on a large curvature by the isolated ventricle method according to I.P. Pavlov (8).

To determine the concentration of VFA in the liquid contents of the abomasum and rumen and in order to select the optimal dose of acids for their subsequent introduction into the isolated abomasum, Basov canulas were applied on the abomasum and rumen of 3 female sheep of the same species. The test animals were kept on a diet, standardized for protein and total nutritional value, containing hay, fodder beets and concentrates. Tests on the absorption of VFA and their salts were carried out during the first half of the day (morning) after a 12 to 14 hour interruption of food intake.

Acid solutions of 0.05 N concentration were introduced into the isolated abomasum in equivalent amounts: 5 ml (15mg) acetic acid, 5 ml (18.5mg) propionic acid, and 5 ml (22mg) butyric acid. The total volume of the acid mixture introduced into the ventricle amounted to 15 ml.

It is known that the concentration of VFA in the contents of the compound stomach is subject to fluctuations under the influence of various factors, and mainly of feeding. On the basis of this fact, a study was made of the absorption of a more concentrated mixture, consisting of 0.1N solutions of the above acids introduced into the isolated abomasum in the same volume as the 0.05N acid mixture.

The mixture of salt solutions, as well as the acid mixture, was introduced into the isolated abomasum in equal amounts, i.e. 5 ml each of a 0.05M salt solution. The total volume of the salt solution was 15 ml, containing, calculated as the acid, 15 mg acetic, 18.5 mg propionic, and 22 mg butyric acid. The prepared 0.05N solution of acetic, propionic and butyric acids had a pH of 3.05, the salt mixture of the same concentration had a pH of 7.80, and the mixture of 0.1N acids had a pH of 2.90. The hydrogen ion concentration (pH) was determined electrometrically with the aid of a calomel and glass electrode with a pH meter type LP-58.

The acid or salt solution, heated to 37°C, was introduced 3 times on the testing day into the isolated abomasum, and was incubated for 10, 30 and 60 minutes, with a 30-minute interval between introductions. Five tests on the absorption of acids and salts were performed on each sheep. To determine the total amount of absorbed acid, samples taken from the abomasum were distilled in a steam distillation apparatus, followed by titration of the distillate with alkali (0.05 and 0.1N solutions) in the presence of phenolphthalein as indicator. The amount of each individual acid or salt absorbed from the mixture of solutions separated after incubation from the isolated abomasum was determined by chromatographic separation of the studied samples on a silica gel column by a slightly modified Isherwood procedure (3).

Results of Studies

In tests performed on 3 sheep with a canula of the rumen and abomasum (Table 1, n=15), it was found that, as the contents of the rumen passed over (transferred) into the abomasum, the total amount of acetic, propionic

and butyric acids present in the abomasum was several times smaller than in the rumen.

TABLE 1

Absorption of VFA in sheep during the passage (transfer) of VFA from the rumen into the abomasum.

1 В 100 мл жидкого содержимого сычуга			2 В 100 мл жидкого содержимого рубца	
3 Кислоты	4 абсолютное количество (в мг)	5 молярное соотношение (в %)	4 абсолютное количество (в мг)	5 молярное соотношение (в %)
6 Масляная	15.25 ± 0.57	14.13	143.66 ± 1.89	17.49
7 Пропионовая	22.74 ± 0.85	25.10	153.89 ± 1.20	22.28
8 Уксусная	44.43 ± 0.41	60.77	334.78 ± 9.47	60.23
9 Общее количество	82.42 ± 1.61	—	632.33 ± 8.08	—

1-In 100 ml of liquid contents in the abomasum
2-In 100 ml of liquid contents in the rumen
3-Acids
4-Absolute amount (in mg)
5-Molar ratio (in %)
6-Butyric acid
7-Propionic acid
8-Acetic acid
9-Total amount

Of the total amount of VFA found in the abomasum 3 hours after feeding, the acetic acid fraction was over 50%, while the remaining amount consisted of propionic and butyric acids.

From the figures given in Table 2, we can see that, during the 10 minute incubation in the abomasum of the mixture of 0.05N acid solutions, up to 29.9% (4.49 mg) acetic acid was absorbed, while the percentage of absorbed propionic acid was higher and amounted to 37.5% (6.94 mg); up to 40,8% (8.97 mg) butyric acid was absorbed, i.e. this acid was more actively absorbed than propionic acid, and even more so than acetic acid.

TABLE 2

Absorption of a mixture of VFA in the isolated Pavlov abomasum.

(1) Количество (в мл)		(5) Время инкубации кислот в сычуге (в мин.)	n	(2) Всосалось кислот								
				(6) масляной			(7) пропионовой			(8) уксусной		
введено в [illegible]	(4) выведено из сычуга раствора (M)			(в мг M±m)	в %	в мг за 1 мин.	(в мг M±m)	в %	в мг за 1 мин.	(в мг M±m)	в %	в мг за 1 мин.
				Смесь 0.05 н. растворов ← Mixture of 0.05 N solutions								
15.0	17.3	10	15	8.97±0.52	40.8	0.90	6.94±0.41	37.5	0.69	4.49±0.39	29.9	0.45
15.0	19.8	30	15	12.99±0.43	59.0	0.43	10.04±0.48	54.2	0.33	6.32±0.49	42.2	0.21
15.0	24.0	60	15	17.00±0.68	77.3	0.28	13.72±0.60	74.1	0.23	10.13±0.39	67.5	0.17
				Смесь 0.1 н. растворов ← Mixture of 0.1 N solutions								
15.0	17.2	10	15	17.76±0.44	40.4	1.78	13.70±0.33	37.0	1.37	9.63±0.42	32.1	0.96
15.0	20.2	30	15	21.89±0.41	49.7	0.73	17.11±0.32	46.3	0.57	12.65±0.30	42.2	0.42
15.0	23.7	60	15	25.73±0.62	58.5	0.43	20.70±0.56	55.9	0.35	14.23±0.42	47.5	0.24

1-Amount (in ml)
2-Acids absorbed
3-Acid mixture introduced into abomasum
4-Solution (M) removed from abomasum
5-Incubation time of acids in abomasum (in min.)
6-Butyric acid
7-Propionic acid
8-Acetic acid

During the 30 and 60 minutes of incubation of the acid mixture in the abomasum the absolute amount of absorbed acids increased, while the ratio of the absorption rates of the acids remained the same as during 10 minutes of incubation. If we compare the absorption of the acid mixture from solutions of different concentrations, we note that, during 10 minutes incubation, no noticeable difference can be detected in the rate of absorption of acetic, propionic and butyric acids from 0.05 and 0.1N solutions.

When a mixture of acids of different concentration is incubated in the abomasum for a longer time (30-60 min.), a predominance of the rate of absorption from the solution of lower concentration (0.05N) is noted. At the same time, it should be noted that, during 30 minutes incubation, the percentage of acetic acid absorption from both solutions was the same (42.2%). The absolute amount of each absorbed acid, naturally, was always higher in the more concentrated solution.

If the absorption of each separate salt from 15 ml of a 0.05M solution of a salt mixture (Table 3) is compared with the absorption figures of an acid mixture, used in the same volume and molarity (Table 2), the following can be noted. During 10 minutes incubation from 15ml of a mixture of a 0.05M solution of salts, 32.2% (7.08 mg) butyrate, 30.0% (5.55 mg) propionate, and 26.1% (3.91 mg) acetate was absorbed in the abomasum, whereas the absorption of each acid was greater during the same period of incubation, namely 40.8 (8.97 mg) butyric, 37.5% (6.94 mg) and 29.9% (4.49 mg) acetic acid was absorbed.

TABLE 3

Absorption of a mixture of 0.05M solutions of salts of VFA in the isolated Pavlov abomasum of sheep..

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
15.0	16.8	10	15	7.08 ± 0.28	32.2	0.71	5.55 ± 0.31	30.0	0.55	3.91 ± 0.29	26.1	[illegible]
15.0	19.0	30	15	11.08 ± 0.50	58.4	0.39	9.13 ± 0.31	49.4	0.30	5.54 ± 0.33	36.9	0.18
15.0	23.7	60	15	15.30 ± 0.47	72.2	0.27	12.51 ± 0.37	67.5	0.21	8.51 ± 0.32	56.7	[illegible]

1-Amount (in ml)
2-Salt mixture introduced into abomasum
3-Solution (M) removed from abomasum
4-Incubation time of salts in abomasum (in min.)
5-Salts absorbed
6-Sodium butyrate
7-Sodium propionate
8-Sodium acetate

If we compare the absorption of Na salts of VFA during 30-60 minutes with the absorption of free VFA during the same time, we find that in this case the absorption of free VFA was more intensive (by 4.8-10.8%). Consequently, free VFA were more actively absorbed by the abomasum wall than their bound forms, namely the sodium salts of these acids.

Although the absolute amount of absorbed acids and salts increased with increasing incubation time in the abomasum, the intensity of absorption, on the contary, decreased. This is confirmed by the following calculations. For example, for butyric acid, from a 0.1N mixture 1.78 mg/min. was absorbed during 10 minutes, 0.73 mg/min. was absorbed during 30 minutes, and 0.43 mg/min. was absorbed during 1 hour, i.e. no linear dependence was noted between the time the acid was present in the ventricle and the degree of its absorption. A similar rule (law) for the intensity of absorption was established for acetic and propionic acids and their salts.

Discussion of Results

The test data have shown that, during passage from the rumen to the abomasum, the total amount of the tested acids present in the abomasum was 7.7 times lower (or smaller by a factor of 7.7) than in the rumen. The decrease in the amount of VFA, as they pass from the rumen into the abomasum, can be explained by their active absorption of these acids by the rumen wall.

We can assume that VFA are transferred (supplied) from the rumen to the abomasum in a considerably greater concentration than is detected in the liquid contents of the abomasum since, according to the data reported by some investigators (15), for example, 60-70% water is absorbed in the omasum, and only the nutrient mass, having a more firm (dense) consistency, enters the abomasum, where gastric juice is secreted, which makes it possible to approximately compensate the liquid lost in the omasum.

The study of the absorption of a mixture of acids, introduced into the isolated abomasum in equimolar amounts, has shown that each acid has a different rate of absorption. Butyric acid had the highest rate of absorption, propionic acid was absorbed at a slightly lower rate, and acetic acid had the lowest rate, i.e. the rate of absorption of VFA increased with increasing molecular weight of these acids.

A similar rule (law) for the absorption of VFA was observed when these acids were separately incubated in the isolated abomasum of sheep (7) and during incubation of an acid mixture in the isolated rumen of cows (1).

The data obtained by us on the absorption of acetic, propionic and butyric acids from a mixture prepared from acid solutions of different concentration (0.05 and 0.1 N mixtures) and introduced into the isolated abomasum in the same volume (15 ml) have shown that, during 30 and 60 minutes incubation, the rate of absorption of VFA, expressed in percent, from the solution of lower concentration was greater than from the solution of higher concentration (0.1 N).

In regard to absorption during a shorter incubation time of the acid mixture (10 min.), no noticeable difference in the rate of absorption was detected.

In all cases, the absolute amount of each absorbed acid was greater in tests where a solution of higher concentration was introduced, which agrees with the data of authors (2) who studied the effect of concentration on absorption. If we compare the rate of absorption of a mixture of VFA and a mixture of sodium salts of these acids, we note that in each time period, free VFA were more actively resorbed by the abomasum wall than their sodium salts.

Our data agree with the observations of V.I. Skorokhod (6) and V.F. Vrakin (1), who studied the absorption of VFA and their sodium salts in the isolated rumen of cattle.

The absorption of sodium salts of VFA followed a less intensive course than that of free acids, but the level of absorption of the salts was often quite close to the absorption of acids.

Data are found in the literature, indicating that the rate of absorption of VFA in the rumen drops considerably with an increase in pH (13,14). At low pH values, the rate of absorption of VFA is present in a free form, in which they are absorbed more rapidly.

Special tests (1) have shown that an increase in the concentration of hydrogen ions in the isolated rumen resulted in a noticeable increase in the absorption of VFA. We have found that the longer the salt or the acid was incubated in the abomasum, the greater was the volume of the extracted (removed) solution in comparison with the amount introduced, whereas its pH dropped, which we must assume, is connected with the secretion of the acid abomasum juice.

The mixture of 0.05 M salt solutions, used for introduction into the isolated abomasum, had a weak alkaline reaction. After the salt mixture was introduced into the abomasum (depending upon the incubation time), the solution removed (extracted) had an acid reaction (pH from 1.00 to 6.50).

The high intensity of absorption of sodium salts of VFA observed in our tests can possibly be explained as due to the secretion by the abomasum of hydrochloric acid, which displaces weak acids from their sodium salts, while acids are more intensively absorbed than salts, as was established in the case of the rumen.

Conclusions

1. The chyme of the abomasum contains a considerable amount of volatile fatty acids, although their concentration in the abomasum is several times smaller than in the rumen. The acids entering the abomasum are present in a definite molar ratio, the highest amount consisting of acetic acid (60.77%), followed by propionic acid (25.10%) and butyric acid (14.13%).

2. Volatile fatty acids (acetic, propionic and butyric), when introduced into the isolated abomasum as a mixture and in equimolar amounts, undergo a very intensive absorption. At various periods of incubation of the acids (10, 30 and 60 min.) and at various concentrations (0.05 and 0.1 N), the rate of absorption decreased in the order: butyric > propionic > acetic acid. The absolute amount of absorbed acids from an acid mixture of higher concentration was greater than from an acid mixture of lower concentration.

3. At the same molarity and volume of solutions, the sodium salts of these acids are absorbed to a lesser extent than the free acids. The absorption of salts from a mixture decreased in the same order as that of acids: butyrate > propionate > acetate.

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ВСАСЫВАНИЕ ЛЕТУЧИХ ЖИРНЫХ КИСЛОТ И ИХ СОЛЕЙ В СЫЧУГЕ ОВЕЦ

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В результате бактериальных процессов в преджелудках жвачных питательные вещества корма расщепляются с образованием ряда веществ, среди которых особое значение придается органическим кислотам с короткой углеродной цепью — летучим жирным кислотам (ЛЖК).

В последние годы отечественными и зарубежными учеными был выяснен ряд особенностей всасывания ЛЖК с некоторых отделов пищеварительного тракта. Было показано, что основные кислоты брожения: уксусная, пропионовая и масляная активно всасываются в месте их образования — рубце и книжке [1, 8, 14, 15]. Однако не все ЛЖК всасывается в преджелудках, значительная часть их может переходить в нижние отделы пищеварительного тракта — сычуг и кишечник [5, 17]. У жвачных животных постоянно всасывающиеся низкомолекулярные кислоты используются для энергетических потребностей организма и синтеза некоторых органических соединений, как глюкоза, жир молока и др. [10, 12, 18].

Сведения о том, как всасываются ЛЖК и их соли в сычуге, или истинном желудке жвачных, до настоящего времени остаются мало выясненными.

Задача настоящего исследования состояла в том, чтобы изучить всасывание ЛЖК и их солей в сычуге овец.

МЕТОДИКА

Опыты по изучению всасывания ЛЖК и их солей проведены на 3 овцематках горно-карпатской породы с изолированными сычужками, сделанными на большой кривизне методом изолированного желудочка, по И. П. Павлову [6].

Для определения концентрации ЛЖК в жидком содержимом сычуга и рубца и с целью подбора оптимальной дозы кислот для последующего введения их в изолированный сычуг 3 овцематкам той же породы на сычуг и рубец были наложены фистулы по Басову. Подопытные животные находились на нормированном по белку и общей питательности рационе, в состав которого входили сено, кормовая свекла и концентраты. Опыты по изучению всасывания ЛЖК и их солей проводили в первую половину дня после 12—14-часового перерыва в приеме корма.

Растворы кислот 0.05 н. концентрации вводили в изолированный сычуг в эквимолярных количествах: 5 мл (15 мг) уксусной кислоты, 5 мл (18.5 мг) пропионовой и 5 мл (22 мг) масляной кислоты. В общей сложности введенная в желудочек смесь растворов кислот составляла 15 мл.

Известно, что под влиянием различных факторов, и в основном кормления, концентрация ЛЖК в содержимом сложного желудка подвержена колебаниям. Исходя из этого, изучалось всасывание более концентрированной смеси, состоящей из 0.1 н. растворов указанных кислот и вводимых в изолированный сычуг в аналогичном объеме, что и 0.05 н. смесь кислот.

Смесь растворов солей, так же как и смесь кислот, вводили в изолированный сычуг в равных количествах: по 5 мл 0.05 *М* раствора соли. Общий объем раствора солей составлял 15 мл, в котором в перерасчете на кислоту содержалось 15 мг уксусной, 18.5 мг пропионовой и 22 мг масляной кислоты. Приготовленный 0.05 н. раствор уксусной пропионовой и масляной кислот имел pH 3.05, смесь солей этой концентрации 7.8

и смесь 0,1 н. кислот 2,9%. Концентрацию водородных ионов (pH) определяли электрометрически с помощью платинового и стеклянного электродов pH метром типа ЛП-5.

Раствор кислот или солей, подогретый до 37°, вводили в изолированный сычуг в день опыта 3 раза и инкубировали на протяжении 10, 30 и 60 мин. с интервалом между введениями 30 мин. На каждой овце проведено 5 опытов по всасыванию кислот и солей. Для установления общего количества всосавшейся кислоты отобранные из сычуга пробы перегонялись в аппарате паровой дистилляции с последующим титрованием отгона щелочью (0,05 и 0,1 н. раствор) в присутствии индикатора фенолфталеина. Количество всосавшейся каждой в отдельности кислоты или соли из смеси растворов, введенных после инкубации из изолированного сычуга, определяли хроматографическим разделением исследуемых проб на силикагелевой колонке, по Инервуду в некоторой модификации [1].

РЕЗУЛЬТАТЫ ИССЛЕДОВАНИЙ

В опытах, выполненных на 3 овцах с фистулой рубца и сычуга (табл. 1, $n = 15$), установлено, что по мере перехода содержимого из рубца в сычуг

Таблица 1

Всасывание ЛЖК у овец по мере перехода из рубца в сычуг

(3) Кислоты	(1) в 100 мл жидкого содержимого сычуга: (4) абсолютное количество (в мг)	(1) в 100 мл жидкого содержимого сычуга: (5) молярное соотношение (в %)	(2) в 100 мл жидкого содержимого рубца: (4) абсолютное количество (в мг)	(2) в 100 мл жидкого содержимого рубца: (5) молярное соотношение (в %)
(6) Масляная	15.25 ± 0.57	14.13	143.66 ± 1.89	17.49
(7) Пропионовая	22.74 ± 0.85	25.10	153.80 ± 1.20	22.28
(8) Уксусная	44.43 ± 0.41	60.77	334.78 ± 9.47	60.23
(9) Общее количество	82.42 ± 1.61	—	632.23 ± 8.68	—

суммарное количество уксусной, пропионовой и масляной кислот в нем было в несколько раз меньше, чем в рубце.

Из общего количества ЛЖК, найденных в сычуге через 3 часа после кормления, на долю уксусной кислоты приходилось более половины, остальное количество составляли пропионовая, затем масляная кислоты.

Из данных табл. 2 видно, что за 10 мин. инкубации в сычуге смеси 0,05 н. растворов кислот уксусная кислота всосалась до 29,9% (4,49 мг), тогда как процент всасывания пропионовой кислоты был выше и составлял 37,5% (6,34 мг); масляная кислота всосалась до 40,8% (8,97 мг), т. е. всасывалась с большей активностью, чем пропионовая, и еще более, чем уксусная кислоты.

За 30 и 60 мин. инкубации смеси кислот в сычуге абсолютное количество всосавшихся кислот увеличивалось, соотношение же скоростей всасывания кислот оставалось таким, как и за 10 мин. инкубации. Сравнивая всасывание смеси кислот из растворов разных концентраций, отмечаем, что за 10 мин. инкубации заметной разницы в скорости всасывания уксусной, пропионовой и масляной кислот от 0,05 и 0,1 н. растворов не выявлено.

При инкубации в сычуге смеси кислот различной концентрации более продолжительное время (30—60 мин.) отмечается преобладание скорости всасывания из раствора меньшей концентрации (0,05 н.). В то же время следует отметить, что за 30 мин. инкубации процент всасывания уксусной кислоты из обоих растворов был одинаковым (42,2%). Абсолютное количество каждой всосавшейся кислоты, естественно, было всегда выше для более концентрированного раствора.

Сравнивая всасывание каждой соли в отдельности из 15 мл 0,05 *М* раствора смеси солей (табл. 3) с данными всасывания смеси кислот, приготовленных в аналогичном объеме и молярности (табл. 2), можно отметить следующее.

За 10 мин. инкубации из 15 мл смеси 0.05 *M* раствора солей в сычуге всосалось 32.2% (7.08 мг) бутирата, 30.0% (5.55 мг) пропионата и 26.1% (3.91 мг) ацетата, тогда как за такой же период инкубации всасывание каждой кислоты было больше. Масляной кислоты всосалось 40.8% (8.97 мг), 37.5% (6.94 мг) пропионовой и 29.9% (4.49 мг) уксусной кислоты.

Если сравнить всасывание натриевых солей ЛЖК за 30—60 мин. со всасыванием свободных ЛЖК за тот же период времени, оказывается, что и в этом случае свободные ЛЖК всасывались более интенсивно (на 4.8—10.8%). Следовательно, свободные ЛЖК всасывались стенкой сычуга более активно, чем их связанные формы — натриевые соли этих кислот.

Хотя абсолютное количество всосавшихся кислот и солей с увеличением времени инкубации в сычуге увеличивалось, интенсивность всасывания, наоборот, снижалась. Это подтверждается следующими расчетами. Например, для масляной кислоты из 0.1 н. смеси за 10 мин. в 1 мин. всосалось 1.78 мг, за 30 мин. — 0.73 и за 1 час — 0.43 мг/мин., т. е. не наблюдалось линейной зависимости между продолжительностью пребывания кислоты в желудочке и степенью ее всасывания. Аналогичная закономерность интенсивности всасывания установлена для уксусной и пропионовой кислоты и их солей.

ОБСУЖДЕНИЕ РЕЗУЛЬТАТОВ

Данные опытов показали, что при поступлении из рубца в сычуг общее количество исследуемых кислот было в 7.7 раза меньше, чем в рубце.

Таблица 1

Всасывание смеси ЛЖК и их солей у взрослого кавловского сычуга

(1) Количество (в мг) в растворе в сычуге смеси кислот	(2) выпито из сычуга раствора (*M*)	(3) Время инкубации в сычуге (в мин.)	*n*	(4) масляной: мг ± m	(4) масляной: %	(4) масляной: мг за 1 мин.	(5) пропионовой: мг ± m	(5) пропионовой: %	(5) пропионовой: мг за 1 мин.	(6) уксусной: мг ± m	(6) уксусной: %	(6) уксусной: мг за 1 мин.
				(1) мг/мин			(2) Всосалось кислот мг/мин					
				Смесь 0.05 н. растворов ← *N/20 solutions* of 0.05 *N* solutions								
15.0	17.3	10	15	8.97 ± 0.52	40.8	0.90	6.94 ± 0.41	37.5	0.69	4.49 ± 0.39	29.9	0.45
15.0	19.8	30	15	12.96 ± 0.43	59.0	0.43	10.04 ± 0.48	54.2	0.33	6.32 ± 0.49	42.1	0.21
15.0	24.0	60	15	17.00 ± 0.68	77.3	0.28	13.72 ± 0.90	74.1	0.23	10.13 ± 0.39	67.5	0.17
				Смесь 0.1 н. растворов ← *N/10 solutions* of 0.1 *N* solutions								
15.0	17.2	10	15	17.76 ± 0.44	40.4	1.78	13.70 ± 0.33	37.0	1.37	9.93 ± 0.42	32.1	0.96
15.0	20.2	30	15	21.89 ± 0.41	49.7	0.73	17.11 ± 0.32	46.3	0.57	12.65 ± 0.39	42.2	0.42
15.0	23.7	60	15	25.73 ± 0.62	58.5	0.43	20.70 ± 0.55	55.9	0.35	17.23 ± 0.42	57.5	0.24

Таблица 3

Всасывание смеси 0,05 *М* растворов солей ЛЖК в изолированном навозном сычуге овец

(1) Количество (в мл)		(3) Выдержка на сухом воздухе солей *М*	(4) Время инкубации солей в сычуге (в мин.)	*n*	(5) Всасываемость солей							
					(6) масляновокислого натрия			(7) пропионовокислого натрия			(8) уксуснокислого натрия	
(2) вводимых в сычуг солей					*μμ* в мг на 1 мин	*μμ* в %	в мг на 1 мин	*μμ* в мг на 1 мин	*μμ* в %	в мг на 1 мин	*μμ* в мг в кг *М* ± *m*	*μμ* в %	в мг на 1 мин
1,5	1,5	16,8	30	15	7,08 ± 0,28	32,2	0,71	5,55 ± 0,35	30,0	0,55	3,91 ± 0,29	26,1	0,39
1,5	1,5	19,5	30	15	11,08 ± 0,50	33,1	0,39	9,13 ± 0,34	29,4	0,30	5,54 ± 0,33	35,9	0,42
1,5	1,5	23,7	60	15	15,90 ± 0,37	72,2	0,27	12,71 ± 0,37	67,5	0,21	8,51 ± 0,32	38,7	0,14

Уменьшение количества ЛЖК по мере перехода из рубца в сычуг объясняется активным всасыванием этих кислот стенкой преджелудков.

Можно допустить, что ЛЖК поступают из преджелудков в сычуг в значительно большей концентрации, чем это обнаружено в жидком содержимом сычуга, так как, по данным некоторых исследователей [15], например, в книжке всасывается 60—70% воды и только более плотная по консистенции пищевая масса поступает в сычуг, где выделяется желудочный сок, который позволяет примерно сбалансировать жидкость, потерянную в книжке.

Изучение всасывания смеси кислот, введенных в изолированный сычуг в эквимолярных количествах, показало, что скорость всасывания каждой кислоты была разной. С наибольшей скоростью всасывалась масляная кислота, с несколько меньшей — пропионовая, затем уксусная кислота, т. е. скорость всасывания ЛЖК увеличивалась с увеличением молекулярного веса этих кислот.

Аналогичная закономерность всасывания ЛЖК наблюдалась при их раздельной инкубации в изолированном сычуге овец [1] и при инкубации в составе смеси в изолированном рубце коров [1].

Полученные нами данные о всасывании уксусной, пропионовой и масляной кислот из смеси, составленной из растворов кислот, имеющих разную концентрацию (смесь 0,05 и смесь 0,1 н.) и введенных в изолированный сычуг в одинаковом объеме (15 мл), показали, что за 30 и 60 мин. инкубации скорость всасывания ЛЖК, выраженная в процентах, из раствора низшей концентрации была выше, чем из раствора большей концентрации (0,1 н.).

Что касается всасывания за более короткий промежуток инкубации смеси кислот (10 мин.), то заметной разницы в скорости всасывания не выявлено.

Во всех случаях абсолютное количество каждой всосавшейся кислоты было больше в опытах с введением раствора высшей концентрации, что согласуется с данными авторов [2], изучавших влияние концентрации на всасывание. Сравнивая скорость всасывания смеси ЛЖК и смеси натриевых солей этих кислот, отмечаем, что свободные ЛЖК за каждый период времени резорбировались стенкой сычуга более активно, чем их натриевые соли.

Наши данные совпадают с наблюдениями В. И. Скорохода [2] и В. Ф. Вракина [3].

которые изучали всасывание ЛЖК и их натриевых солей в изолированном рубце крупного рогатого скота.

Всасывание натриевых солей ЛЖК происходило с меньшей интенсивностью, чем свободных кислот, но по уровню всасывания соли нередко приближались к всасыванию кислот.

В литературе имеются данные, указывающие на то, что скорость всасывания ЛЖК значительно снижается в рубце при повышении pH [12, 11]. При низких pH скорость всасывания ЛЖК увеличивалась: в кислой среде значительное количество ЛЖК существует в свободной форме, в которой они быстрее всасываются.

Специальные опыты [1] показали, что увеличение концентрации водородных ионов в изолированном рубчике приводило к заметному увеличению всасывания ЛЖК. Нами установлено, что чем больше инкубировался в сычуге раствор соли или кислоты, тем объем извлеченного раствора увеличивался в сравнении с введенным количеством, а показатель его pH снижался, что, надо полагать, было связано с секрецией кислого сычужного сока.

Смесь 0.05 *M* растворов солей, применявшаяся для введения в изолированный сычужок имела слабощелочную реакцию. После введения в сычуг смеси солей (в зависимости от времени инкубации) выведенный раствор имел кислую реакцию (pH от 1.00 до 6.50).

Высокая интенсивность всасывания натриевых солей ЛЖК в наших опытах, возможно, объясняется секрецией сычужком соляной кислоты, которая вытесняет слабые кислоты из их натриевых солей, а кислоты, как установлено на примере рубца, всасываются более интенсивно, по сравнению с солями.

ВЫВОДЫ

1. В химусе сычуга содержится значительное количество летучих жирных кислот, хотя концентрация их в сычуге в несколько раз меньше, чем в рубце. Поступающие в сычуг кислоты находятся в определенных молярных соотношениях, в которых наиболее представлена уксусная кислота (60.77%), затем следует пропионовая (25.10%) и масляная (14.13%) кислоты.

2. Летучие жирные кислоты — уксусная, пропионовая и масляная, — введение в изолированный сычуг в смеси и в эквимолярных количествах, всасываются очень интенсивно. За разные периоды инкубации кислот (10, 30 и 60 мин.) и из разной концентрации (0.05 и 0.1 н.) по скорости всасывания кислоты располагаются в следующей последовательности: масляная > пропионовая > уксусная. Из смеси кислот более концентрированного раствора абсолютное количество всосавшихся кислот было большим, чем из смеси раствора менее концентрированного.

3. При одинаковых молярности и объеме растворов натриевые соли кислот всасываются слабее, чем свободные летучие жирные кислоты. Порядок всасывания солей из смеси оставался таким, каким он был установлен для всасывания кислот: бутират > пропионат > ацетат.

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ABSORPTION OF VOLATILE FATTY ACIDS AND THEIR SALTS IN SHEEP ABOMASUM

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Experiments were carried out on sheep with the Basov's canula of the rumen and abomasum. The liquid part of the abomasum showed the quantity of volatile fatty acids (VFA) to be 7.7 times less in comparison with the rumen.

The acetic acid in all the quantity of VFA found in the abomasum represented 60.77 molar per cent, propionic — 25.10 and butyric acid — 14.13 respectively.

Experiments on the isolated abomasum by Pavlov's method showed that the equimolar quantity of VFA introduced in the abomasum is very intensively absorbed by the abomasum walls. The rate of absorption depends upon the kind of acids, the succession being as follows: butyric > propionic > acetic.

The acid mixture of a more concentrated solution in higher quantity of absorbed acids, and on the contrary, a less concentrated solution of mixture resulted in a lower quantity of absorbed acids.

Sodium salts of the acids are absorbed less intensively as compared to the VFA. The succession of absorption from the mixture remained the same, i. e. butyric > propionic > acetic.

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RANGE-FINDING TOXICITY DATA: LIST IV

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SINCE the appearance of our previous communications on the range-finding test[1] additional data from our work have become available for presentation. They are listed in Tables 1 and 2. All materials named either are being handled in commercial quantities or have been evaluated for specific commercial applications within the past few years. A few are known by trade names not suggested by the chemical names given here.

There has been no change in methods or in presentation of data since the last paper. Two changes are contemplated. The rabbit-eye-injury test is unrealistic in that it exaggerates the hazard from certain materials. It is desirable to add to the present test, which determines the least amount of chemical causing corneal injury of an arbitrary severity, some expression for the rate of healing of the injury. Background studies for this addition have not yet been made.

In Table 2 appears no material which caused microscopic lesions where some other evidence of injury was not found at the same or a lower dose level. Accordingly, we tentatively expect to drop microscopic study of tissues from subacute dosage series at the range-finding level on the ground that the technic is not sufficiently sensitive to justify its cost.

None of our earlier papers have pointed out that, except in rare cases, all single oral doses are given in this laboratory to animals which have not been fasted overnight. In the few instances where we have made comparisons, the $L.D._{50}$ determined on fasted rats is lower than that on animals not fasted by only some 20 or 30%, a difference on the borderline of statistical significance and not great enough to influence evaluation of practical hazards.

From Mellon Institute of Industrial Research, University of Pittsburgh.

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TABLE 1.—*Range-Finding Data on Toxicity*

Material Studied	Single-Dose Oral Toxicity for Rats (R. F. L. D_{50} After 14 Days), Gm./Kg.*	Penetration of Rabbit Skin (R. F. L. D_{50} After 14 Days), Ml./Kg.*	Saturated Vapor Inhalation by Rats; Maximum for No Death	Inhalation of Known Vapor Concentration by Rats: Concentration, PPM	Time, Hr.	Mortality in 14 Days	Primary Skin Irritation on Rabbits†	Eye Injury in Rabbits from Fluid‡
Acetaldehyde	1.86 (1.62-2.21)	.....	2 min.§	5,000	8	0/6	1	1
				16,000	8	0/6		
Acetic acid	3.73 (3.29-3.85)	.....	.....	16,000	4	1/6	3	10
				11,000	4	0/6		
Acetic anhydride	1.75 (1.43-2.13)	.....	5 min.	2,000	4	0/6	2	9
				8	4	1/6		
Acrolein (acrylaldehyde)	0.046 (0.036-0.058)	.....	4 min.§	.....	.....	.....	10	10
2-Ethylhexyl 2-nitropropyl ether	0.32	0.36	8 hr.	.....	.....	.....	6	9
2-Aminoethanol	3.06	1.10 (0.72-2.00)	8 hr.	.....	.....	.....	6	9
1-Aminopropylmorpholine	3.96	1.23 (0.94-1.63)	8 hr.	.....	.....	.....	6	9
Benzotrichloride (α,α,α-trichlorotoluene)	0.9	.....	30 min.	125	8	5/6	10	10
Benzyl alcohol	0.10 (0.85-1.37)	.....	2 hr.	1,000	8	3/6	6	9
1,3-Butanediamine	1.35	0.43 (0.31-0.60)	8 hr.	.....	.....	.....	1	5
1,3-Butanediol	22.8 (21.8-23.9)	.....	8 hr.	.....	.....	.....	1	5
n-Butanol	4.36 (3.96-4.78)	.....	8 hr.	.....	.....	.....	1	5
n-Butyl acrylate	3.73 (2.49-5.21)	3.36 (2.40-4.72)	30 min.	1,000	8	5/6	3	8
2-Butyl-1-octanol	12.9 (9.9-17.0)	.....	2 hr.	.....	.....	.....	1	1
Butyraldehyde	3.79 (3.54-4.25)	.....	5 min.	5,000	8	1/6	3	8
Butyric acid	2.04 (2.04-2.50)	.....	8 hr.	.....	.....	.....	1	1
2-Chloroacrylidene 3,3-diacetate	0.32 (0.21-0.50)	0.58 (0.08-1.10)	2 hr.	8	8	3/6	10	10
2-Chloro-1,1,3-triethoxypropane	1.32	8	2 hr.	2,000	8	3/6	1	1
				125	8	0/6		
2-Chloroethyl acrylate	0.18	.....	15 min.	375	8	0/6	10	10
2-Chloro-1,1,2-trifluoroethyl methyl ether	5.13 (3.91-6.71)	0.2	5 min.	.....	.....	.....	1	1
2-Butenylphenol (mixed isomers)	0.41 (0.29-0.96)	.....	8 hr.	.....	.....	.....	10	10
Cumene (isopropylbenzene)	2.91 (2.55-3.32)	12.3 (7.6-19.7)	1 hr.	8,000	4	4/6	1	1
Decahydronaphthalene	4.17 (3.29-5.16)	5.96 (3.81-9.06)	2 hr.	500	4	4/6	1	1
Decanol (mixed isomers)	9.80 (7.47-12.8)	8.76 (2.29-5.78)	8 hr.	.....	.....	.....	1	1
Dibutyl adipate	12.9 (9.54-17.0)	20	8 hr.	.....	.....	.....	1	1
Dibutyl maleate	8.58 (6.12-11.9)	15.9 (7.32-28.2)	8 hr.	.....	.....	.....	1	1
Dichloroacetic acid	2.82	0.51	5 hr.	.....	.....	.....	1	1
Dichloroacetic anhydride	2.82	0.17	5 hr.	.....	.....	.....	1	1
Dichloroacetyl chloride	2.45 (1.85-3.23)	0.65 (0.73-0.81)	8 min.	2,000	8	5/6	10	10
1,4-Dichlorobutene	0.99 (0.64-0.100)	0.62 (0.47-0.81)	8 min.	62	8	1/6	10	10
2,2-Dichloroisopropyl ether	0.24 (0.22-0.27)	3.01 (1.75-5.04)	.....	1,000	8	1/6	10	10
2,4-Dichlorophenoxy ethanol	1.07 (0.76-1.45)	0.42 (0.25-0.71)	8 hr.	.....	.....	.....	1	1
α,β-Dichloropropionaldehyde	0.13 (0.11-0.19)	0.005 (0.051-0.112)	2 min.§	16	8	1/6	10	10
Diethylamine	0.54 (0.35-0.83)	0.52 (0.38-1.59)	5 min.§	1,000	8	3/6	10	10
				62	8	0/6		
N,N-Diethylaminoacetonitrile	0.15 (0.10-0.24)	0.33 (0.24-0.52)	8 min.	125	8	0/6		
N-Diethylaminoethyl chloride	0.317 (0.211-0.997)	0.3	2 min.	.....	.....	.....	1	1
Di(2-ethylhexyl) adipate	0.11 (0.7-0.11)	16.3 (14.5-18.6)	5 hr.	.....	.....	.....	1	1
Diethyl succinate	8.53 (8.11-1.6)	.....	8 hr.	.....	.....	.....	1	1
Di(2-methoxyethyl) maleate	8.31 (3.85-8.74)	1.04 (1.06-3.55)	8 hr.	.....	.....	.....	1	1
2-Dimethylaminoethanol	2.34 (2.76-2.42)	1.37 (1.71-1.55)	8 hr.	.....	.....	.....	1	1
2,6-Dimethylheptanol, o-t-p (monylphenol)	1.02 (1.09-2.56)	2.11 (1.52-2.59)	1 hr.	.....	.....	.....	1	1
Diethylhexylphenylphosphite (DP-diphenol)	4.16 (3.73-4.8)	3	.....	.....	.....	.....	1	1
Dimethyl sulfate	0.15 (0.12-0.45)	0.5	5 hr.	.....	.....	.....	1	1
1,3-Diphenoxy-2-propanol	1.45	16.8	2 min.	50	8	5/6	1	1
			1 hr.	.....	.....	.....	1	1

Ethoxypropyl acrylate	0.82 (0.62-1.07)	1.41	1 hr.	2,000	4	1/6	4	4
Ethylene triethylene glycol	10.5 (9.5-11.5)	8	.....	..	..	...	1	1
2-Ethylhexyl acrylate	4.3 (4.0-5.5)	5.50 (2.98-11.2)	4 hr.	..	..	...	5	2
Ethyl butyl ether	1.37 (1.24-2.40)	.....	5 min.	1,000	4	0/6	2	2
α-Ethylbutyraldehyde	3.98 (3.34-4.74)	.....	5 min.	8,000	4	5/6	..	..
Ethylenediamine	1.16 (0.98-1.37)	0.73 (0.64-0.82)	.....	{2,000 {4,000	8 8	0/6} 6/6}	6	8
Ethyl β-ethoxypropionate	5.6	10	8 hr.	..	..	...	2	3
α-Ethylene glycol diol	3.73 (2.13-5.74)	5.04 (3.10-7.36)	.....	1,000	4	1/6	5	2
2-Ethylhexyl acrylate	5.98	8.48	8 hr.	..	..	...	5	1
2-Ethylhexyl chloride	7.31 (4.04-11.5)	.....	1 hr.	2,000	4	4/6 ‡	4	1
2-Ethylbutyraldehyde acid	5.06 (2.02-12.2)	2.75 (1.49-5.08)	8 hr.	..	..	...	2	9
3-Heptanol	1.87 (1.24-2.60)	4.26 (2.26-8.16)	4 hr.	..	..	...	3	5
n-Hexanol	4.50 (4.03-5.24)	3.10 (2.02-4.77)	8 hr.	..	..	...	3	9
n-Hexoxyethoxyethanol	3.92 (3.75-6.46)	1.50 (1.07-2.10)	8 hr.	..	..	...	4	8
2-Hydroxyethyl acrylate	1.07 (0.77-1.41)	1.01 (0.58-1.49)	1 hr.	500	4	5/6	3	5
n-Hexyl benzoate	12.3 (11.3-13.5)	21	8 hr.	..	..	...	3	1
N-Hydroxyethyl diethylenetriamine	1.92 (2.75-6.46)	2.52 (1.70-3.73)	8 hr.	..	..	...	2	5
Isopropylamine	0.82 (0.67-1.07)	0.55 (0.34-1.02)	2 min.§	{4,000 {8,000	4 4	0/6} 6/6}	6	10
Isopropyl benzoate	3.73 (2.13-5.73)	20	1 hr.	..	..	...	3	1
2-Methyl-1,4-butanediol	5.40 (4.79-6.23)	2.62 (2.39-2.89)	8 hr.	..	..	...	1	5
4-Methyl-2-pentanol (methylisobutylcarbinol)	2.50 (2.26-2.87)	3.56 (2.72-4.76)	2 hr.	2,000	8	5/6	2	5
α-Methylbenzylamine	0.84 (0.37-1.31)	0.78 (0.42-1.45)	8 hr.	..	..	...	6	9
α-Methylbenzylamine, N-hydroxyethyl	2.83 (1.64-4.87)	1.54 (0.91-2.61)	8 hr.	..	..	...	1	8
2-Methyl-1-buten-3-one	0.18	0.23 (0.07-0.77)	2 min.§	125	4	5/6	3	10
2-Methyl-5-ethylpyridine	1.54 (1.22-1.94)	3.8	1 hr.	1,000	4	5/6	6	9
4-Methyl-2-pentanone	2.08 (1.91-2.27)	.....	15 min.	{2,000 {4,000	4 4	0/6} 6/6}	..	..
N-Methyl-N-hydroxyethylaniline	2.63 (1.64-4.87)	3.25 (2.11-4.99)	8 hr.	..	..	...	3	9
Methyl silicate	.....	17	5 min.	{125 {250	4 4	0/6} 6/6}	..	9
Oxathiane (thioxane)	2.83	.....	3 hr.	8,000	4	5/6	2	4
Phenyl xylyl ketone	4.92 (3.75-6.46)	20	8 hr.	..	..	...	1	1
α-Picoline	1.41	0.41 (0.27-0.63)	1 hr.	{2,000 {4,000	4 4	0/6} 6/6}	4	8
Propionaldehyde	1.41 (0.96-2.08)	5.04 (3.40-7.46)	2 min.	8,000	4	5/6	2	5
Propionitrile	0.039 (0.029-0.051)	0.21 (0.15-0.30)	2 min.§	500	4	2/6	1	4
Pyridine	1.58 (1.42-1.77)	.....	30 min.	4,000	4	5/6	3	7
Quinaldine	1.23 (0.94-1.62)	1.87 (1.34-2.60)	4 hr.	..	..	...	2	9
Quinoline	0.46 (0.21-0.99)	0.54 (0.35-0.83)	8 hr.	..	..	...	4	8
Iso quinoline	0.26	0.50 (0.38-0.91)	.....	..	..	...	5	9
1,1,3,3-Tetraethoxypropane	3.73 (2.68-5.21)	.....	8 hr.	..	..	...	1	2
Tetraethylene glycol dibutyl ether	6.5	10	15 min.	..	..	...	2	6
Tetrahydronaphthalene	2.86 (2.58-3.18)	17.3 (14.5-20.6)	8 hr.	..	..	...	4	1
Tri(2-chloroethyl) phosphate	1.41 (0.96-2.08)	.....	8 hr.	..	..	...	2	1
1,3,3-Triethoxy-1-propene	2.46 (1.88-3.23)	0.37 (0.24-0.57)	2 hr.	250	4	1/6	5	5
1,3,3-Triethoxy propane	1.6	8	8 hr.	..	..	...	4	8
Triethoxymethane (triethyl orthoformate)	7.06 (5.78-8.63)	20	1 hr.	4,000	8	3/6	2	1
Triethylamine	0.46 (0.25-0.83)	0.57 (0.36-0.90)	.....	1,000	4	1/6	2	9
Trimethylnonanone	8.47 (7.18-9.99)	11.0 (9.12-13.0)	4 hr.	..	..	...	3	1
Vinyl butyrate	8.36 (6.12-11.9)	.....	30 min.	4,000	4	3/6	1	1

* The figures in parentheses show the limits of ± 1.96 standard deviations calculated by the method of Thompson; absence of parentheses marks L. D_{50} obtained graphically.

† The grade of primary irritation hazard given is explained by Smyth, Carpenter and Weil.[1c]

‡ The grade of eye hazard given is explained by Carpenter and Smyth.[2]

§ The inhalation time shown killed all rats.

‖ The data were previously published by Smyth, Carpenter and Weil.[1c]

TABLE 2.—*Range-Finding Data on Subacute Oral Toxicity*

Materials Administered in the Food to Groups of Ten Rats for Thirty Days on Each Dose Level Unless Noted Otherwise

Material Studied	Maximum Dose, Gm./Kg.	Minimum Dose, Gm./Kg.	Least Daily Dose Causing Symptom, Gm./Kg. Reduced Growth	Reduced Appetite	Altered Liver or Kidney Weight	Microscopic Lesions †	Death	Maximum Dose Having No Effect Gm./Kg.	Single-Dose Oral L_{50} Gm./Kg.‡
Acrolein *	0.0015	0.0017	....	0.0015	0.0015	.....	.....	0.00017	0.046 (0.039-0.054)
Alkol	1.58	0.326	1.58	.....	.....	.....	.....	0.43	2.18 (2.0-2.38)
Allyl alcohol *	0.0097	0.0018	....	0.0097	.....	0.0097	0.0097	0.0040	0.064 (0.050-0.074)
1,3-Butanediol §	5.20	0.32	....	.....	.....	.....	.....	5.00 ‡	22.8 (21.8-23.9)
"Cellosolve" (2-ethoxyethanol) §	1.89	0.052	0.74	0.74	0.74	0.74	1.89	0.21	3.00 (2.51-3.59)
Dichloral urea	5.33	0.012	0.18	5.33	0.18	2.55	2.55	0.018	32 RF
2,4-Dichlorophenoxy ethyl sulfate, sodium	0.66	0.011	0.66	.....	0.20	.....	0.66	0.017	1.41 RF
Diethanolamine (2,2'-iminodiethanol) ‖	0.68	0.0051	....	0.68	0.000	0.17	0.17	0.020	1.82 (1.26-2.99)
Di(2-ethylhexyl) adipate §	4.71	0.16	2.92	2.92	2.92	2.92	4.71	0.61	9.11 (7.28-11.4)
Di(2-ethylhexyl) tetrahydrophthalate §	2.67	0.058	2.67	.....	0.84	.....	.....	0.19	111 RF
2-Dimethylaminoethanol (dimethylethanolamine) ‖	0.89	0.015	....	.....	0.89	.....	.....	0.18	2.31 (2.26-2.42)
2,4-Dimethyl-2-methylene-1,3,4-thiodiazolidine thione	0.10	0.0025	0.10	0.10	0.039	.....	.....	0.010	0.59 (0.14-0.22)
Dimethyl methylene diphenol-p,p' ‖	0.52	0.002	....	.....	.....	.....	.....	0.32 ‡	4.04 (3.73-4.53)
Dimethyl methylene diphenol-p,p', diglycidyl ether	0.35	0.014	....	.....	.....	.....	.....	0.35 ‡	8.14 (7.29-9.14)
Ethylene chlorhydrin (2-chloroethanol)*	0.012	0.0024	....	.....	0.012	.....	.....	0.0024	0.089 (0.067-0.117)
Ethylenediamine *§	0.31	0.025	0.31	0.12	0.31	0.12	0.12	0.020	1.10 (0.99-1.37)
2-Ethyl-1,3-hexanediol §	0.70	0.20	0.70	.....	.....	.....	.....	0.48	2.71 (2.52-2.93)
2-Ethylhexanoic (diethyl 2-ethyl hexanoate)	1.53	0.10	....	.....	1.53	.....	.....	0.44	40 RF
Glycidyl sorbate dimer	0.27	0.012	0.27	.....	0.012	0.27	0.27	0.012 ¶	5.75 (5.28-6.25)
2-Methyl-2,4-pentanediol (4)	0.31	0.013	....	.....	.....	.....	.....	0.31 ‡	4.70 (4.27-5.20)
Monoethanolamine (2-aminoethanol) §	2.67	0.10	....	.....	0.64	1.28	1.28	0.32	2.74 (2.39-3.15)
Monoisopropanolamine §	2.22	0.14	....	.....	2.22	.....	.....	0.69	4.23 (3.89-4.57)
Tetrabutyl thiodisuccinate §	10.8	0.26	10.8	.....	1.08	10.8	10.3	0.26	100 RF
1,2,3,4-Tetrathia-6,9-diazecane-5,10-dithione	0.55	0.04	0.55	.....	0.17	.....	.....	0.04	8.53 (6.13-11.9)
Triethanolamine §	2.01	0.0050	1.27	.....	0.17	0.73	0.73	0.03	9.11 (8.46-9.82)

* The dose was administered in the drinking water. In this case appetite is judged on the basis of milliliters of water consumed.

† The dose producing microscopic lesions in liver, kidney, spleen or testis of any rat.

‡ When L_{50} was derived from an advanced test, the range of 1.96 times the standard deviation is shown in parentheses. The letters "RF" refer to data from a range-finding test.

‡ The dose shown is the maximum which was administered, and it caused no symptoms.

§ Doses administered for 60 days.

¶ The dose shown is the minimum administered, and it caused some symptoms.

STUDIES OF CHRONIC INTOXICATIONS ON ALBINO RATS

III. ACETIC AND FORMIC ACIDS

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INTRODUCTION

The immediate occasion for investigating these acids was the question of the safety of the use of formic acid for the preservation of foods. The supposed safety of acetic acid for similar purposes made this an admirable measure of comparison; so that parallel experiments were carried out with both acids. However, the investigation was planned with a view to possible wider applications.

The effects of continued use of acetic acid have not been experimentally studied. It seemed of interest to learn whether the continued ingestion of considerable quantities of acid influenced digestion and metabolism, for instance by direct effects on the digestive tract, which is so largely controlled by the acid and alkaline reactions.

Acetic acid is so readily oxidized that it would probably not affect the acid-base equilibrium of the tissues. The oxidation of formic acid is less complete (Fleig), and might therefore conceivably alter the equilibrium, and this would probably be reflected in growth.

Formic acid has a further interest as an oxidation product of methyl alcohol (Pohl). Chronic or subacute poisoning with formic acid, if it exists, might therefore throw some light on chronic or subacute methyl alcohol poisoning.

Finally, some fifteen or eighteen years ago, formic acid and formates were loudly proclaimed as a sort of elixir of youth, destined to redeem the world, and to confer on men the indefatigability of insects. This action, if it existed, should be reflected in the nutrition and growth.

The interesting history of this fantastic propaganda as well as its scientific refutation, are given in the comprehensive paper of Fleig, 1907. He showed the remarkable tonic effects to be merely imaginary. The descriptions of his experiences are worth reading; for they apply equally to more modish tonics and rejuvenants; especially interesting is the description of the asthenic patient (page 215) who recovered his vigor on sodium chlorid, that he mistook for the more advertised formate.

Fleig's paper also contains the scientific data as to formates, of which his own are the most important. He reviews the literature so thoroughly that it is not necessary to enter further into that subject; except to abstract very briefly the data that apply to the present investigation. There seems to have been very little scientific work with formates since Fleig.

METHODS

The experiments were conducted as described in the first and second papers of this series; the acids being added to the drinking water, so that they were consumed continuously.

Dosage. This is determined by the concentration of the drug and by the thirst of the animals. The mean doses are shown in table 1. The weekly variations appear unimportant. They could be calculated from the curve of fluid consumption.

For the preservation of food, 0.1 to 0.15 per cent of free formic acid is said to suffice (Lebbin). This falls within the limit of the concentration (0.01 to 0.5 per cent) of the present experiments.

Lebbin administered to men for four weeks, daily doses of 0.5 gram of formic acid per kilogram of body weight, without any effects. This would correspond to about 8 mgm. per kilogram, which is our smallest dosage. Our largest dosage is 45 times as great. Lebbin also tried doses larger than 0.5 gram. They produced only local actions, analogous to acetic acid. In rabbits, continued administration of either formic or acetic acid damaged the kidneys.

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Of sodium formate. Fleig found the acute toxic dose for dogs to be 3 grams per kilogram by vein, 4 grams per kilogram by mouth; this would correspond to 250 grams for man; i.e., the toxicity of the formate is of the same order as that of sodium chlorid. Men consumed daily doses of 10 grams of sodium formate, equivalent to 150 mgm. per kilogram, for some time without any harm. Eppinger observed hematuria in one out of three patients taking 3 to 4 grams per day; but this looks like an accidental coincidence.

TABLE 1

Dosage of acetic and formic acids

CONCENTRATION OF ACID	EXPERIMENT NUMBER	NUMBER OF ANIMALS IN EXPERIMENT	DURATION	MEAN DOSAGE, MILLIGRAMS OF ACID PER KILOGRAM OF RAT PER DAY	
Acetic acid					
per cent			*weeks*		
0.01	56	6	11	8.2	(5.7–11.3)
0.01	107	3	9	10.9	(9.6–12.9)
0.1	63	6	15	81.0	(63.0–95.0)
0.25 (after 10 weeks of 0.01)	5695	4	15	210.0	(79.0–390.0)
0.5 (after 16 weeks of 0.1)	6305	3	9	360.0	(310.0–710.0)
Formic acid					
0.01	57	6	11	8.2	(5.6–12.1)
0.01	108	3	14	10.25	(8.4–14.3)
0.1	64	6	15	90.0	(80.0–131.0)
0.25 (after 12 weeks of 0.01)	5796	4	15	160.0	(120.0–210.0)
0.5 (after 17 weeks of 0.1)	6406	3	9	360.0	(290.0–670.0)

CONSUMPTION OF FLUID

Table 2 gives the mean daily consumption of fluid in each experiment; as well as the mean for the first, middle and last third of each experiment, so as to indicate the influence of the continuance of the experiment.

Table 2 shows that there are no distinctive differences in the fluid consumption as between acetic or formic acids, or the different concentrations, or the different periods. It may there-

fore be concluded that neither acetic nor formic acid affect the consumption of fluid when added to the drink in concentrations up to at least 0.5 per cent.

TABLE 2

Mean daily consumption of fluid

ACIDS	EXPERIMENT NUMBER	DURATION	FLUID CONSUMPTION (CUBIC CENTIMETERS PER KILOGRAM OF RAT)			
			Entire period	First third	Middle third	Last third
		weeks				
Acetic acid, 0.01 per cent	56	11	82.0 (57–113)	104	80	80
Acetic acid, 0.01 per cent	107	9	109.0 (96–129)	106	112	107
Acetic acid, 0.1 per cent	63	15	81.0 (62–95)	88.5	69	80
Acetic acid, 0.25 per cent	5695	15	90.0 (74–155)	106	88	90
Acetic acid, 0.5 per cent	6305	9	71.0 (63–142)	71	67	86
Formic acid, 0.01 per cent	57	11	82.0 (56–121)	101	75	62
Formic acid, 0.01 per cent	108	14	102.5 (84–125)	122	89	99
Formic acid, 0.1 per cent	64	15	90.0 (80–109)	86	89	92
Formic acid, 0.25 per cent	5796	15	64.0 (49–85)	64	62	66
Formic acid, 0.5 per cent	6406	9	72.0 (58–135)	73	58	82
Formic acid, 0.75 per cent	138	2	127.0 (116–138)			

DIURETIC ACTION

The fact that formic acid does not increase the consumption of fluid implies that it does not act as a diuretic. According to Fleig, formates had considerable reputation as diuretics; and he quoted from Huchard several cases of marked diuresis following the administration of formates to cardiac and renal patients. If these were not accidental coincidences, they must have been due to the alkaline element of the formates. Fleig himself attributes the supposed diuresis to renal vasodilation that he observed on intravenous injection of sodium formate in animals; but this renal effect was evidently due to hydremic plethora from the injection of the solution.

EFFECTS ON GROWTH

The data are shown in table 3 and in figures 1, 2 and 3. It may be recalled that in non-medicated groups of rats, the variation from the standard growth do not exceed −1 to +1.8 per cent per week. The last column of table 3 shows that both

TABLE 3

Effects of acetic and formic acid on growth

DRUG AND CONCENTRATION	DOSAGE, CUBIC CENTIMETERS PER KILOGRAM OF RAT PER DAY	EXPERIMENT NUMBER	DURATION	OBSERVED WEIGHT	NORMAL WEIGHT	DIFFERENCE IN GRAMS	DIFFERENCE IN PER CENT OF NORMAL WEIGHT	DIFFERENCE PER CENT PER WEEK
			weeks					
Acetic acid, 0.01 per cent	8.2	56	11	175	250	−75	−30.0	−2.7
Acetic acid, 0.01 per cent	10.9	107	9	232	230	+2	+0.43	+0.04
Acetic acid, 0.1 per cent	81.0	63	15	304	295	+9	+3.2	+0.21
Acetic acid, 0.25 per cent (after 10 weeks of 0.01 per cent)	210.0	5695	5	209	212	−3	−1.4	−0.28
			15	215	258	−43	−16.0	−1.06
Acetic acid, 0.5 per cent (after 16 weeks of 0.1 per cent)	360.0	6305	1	264	312	−48	−15.0	−15.0
			5	270	315	−45	−14.0	−2.8
			9	240	319	−79	−24.0	−2.6
Formic acid, 0.01 per cent	8.2	57	10	199	190	+9	+4.6	−0.46
			11	162	192	−30	−15.0	−1.3
Formic acid, 0.01 per cent	10.25	108	14	160	177	−17	−9.0	−0.63
Formic acid, 0.1 per cent	90.0	64	15	275	282	−7	−2.0	−0.13
Formic acid, 0.25 per cent (after 12 weeks of 0.01 per cent)	160.0	5796	2	165	168	−3	−1.8	−0.9
			4	222	175	+47	+26.0	+6.4
			10	235	191	+44	+23.0	+2.3
			15	216	197	+19	+9.0	+0.6
Formic acid, 0.5 per cent (after 17 weeks of 0.1 per cent)	360.0	6406	9	180	295	−115	−56.0	−6.7

acetic and formic acids, in concentrations of 0.1 and 0.25 per cent are generally within these limits, and therefore had no effect on growth. With each acid, one experiment with 0.01 per cent also comes within the limits; and one experiment exceeds the

limits. In view of the negative results with 25 times this concentration, this experiment was evidently a coincidence.

By doubling the concentration, that is, with 0.5 per cent acids, and a dosage of 0.36 cc. per kilogram, both acids produced immediate and progressive check of growth, averaging 2.6 per cent per week for acetic acid, and 6.7 for formic acid or total

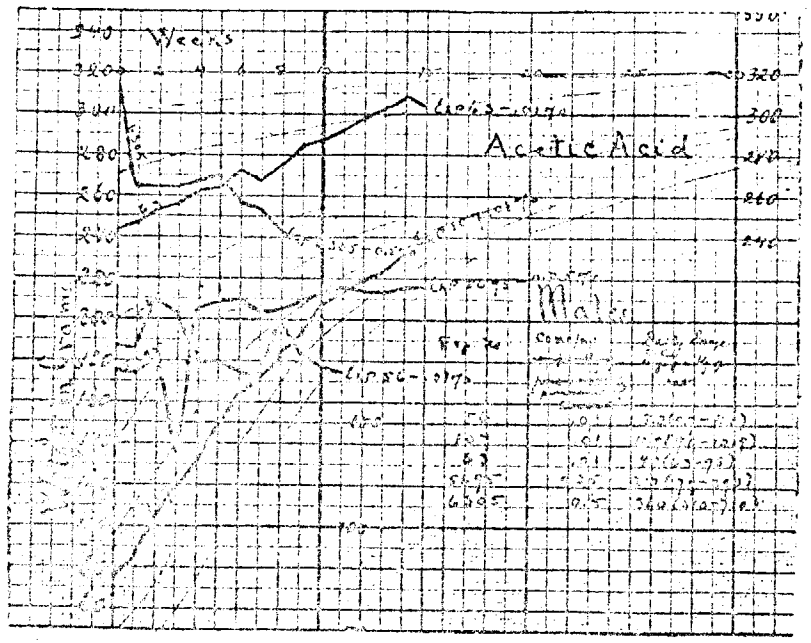

FIG. 1. EFFECT OF ACETIC ACID ON GROWTH

The heavy lines represent the growth of the alcohol rats; the light line represents the standard normal growth curves.

deficits, in the weight, of 24 per cent for acetic acid and 58 per cent for formic acid. The difference between the two acids is evidently accidental; for similar differences, but in the opposite direction, occur with the lower concentrations.

It may therefore be concluded that formic and acetic acid are quantitatively alike in their influence on growth. Neither has any effect in fifteen weeks when the concentration in the drink

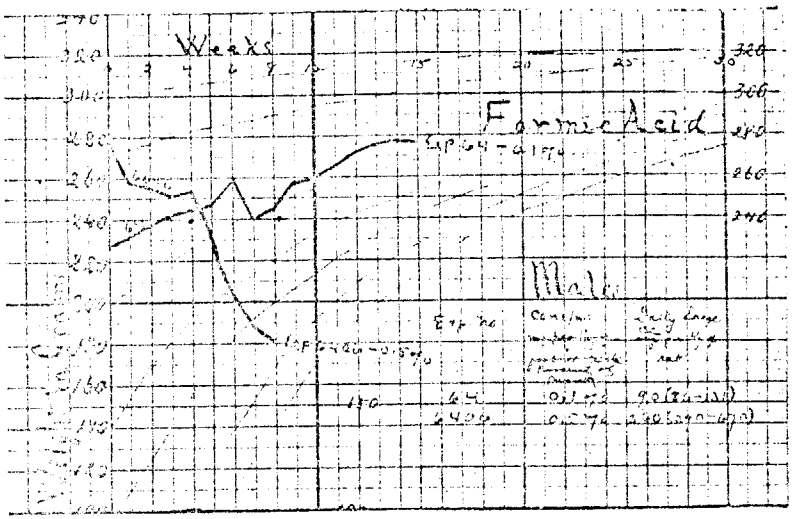

FIG. 2. FORMIC ACID ON GROWTH OF MALE RATS

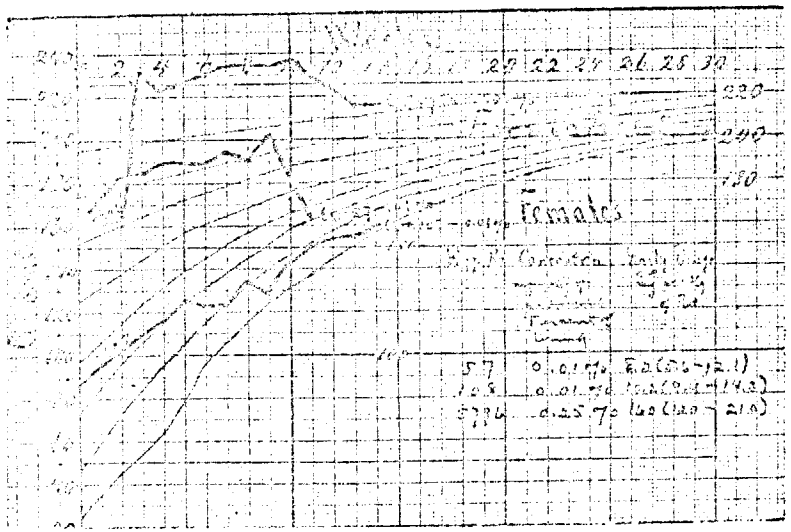

FIG. 3. FORMIC ACID ON GROWTH OF FEMALE RATS

does not exceed 0.25 per cent, with the daily dosage 0.2 cc. per kilogram of rat.

With a concentration of 0.5 per cent and daily dosage of 0.36 cc. per kilogram, growth is definitely checked from the start of the administration.

This does not depend on any change of the total fluid consumption, since this is not materially altered.

EFFECTS ON FOOD CONSUMPTION

These are shown in table 4 and figures 4 and 5.

In interpreting the data of table 4, it must be remembered that the extreme variation of unpoisoned rats from the standard

TABLE 4

Food consumption

ACIDS	EXPERIMENT NUMBER	DURATION OF EXPERIMENT	GROWTH, MEAN DIFFERENCE FROM NORMAL STANDARD PER CENT PER WEEK	FOOD CONSUMPTION, MEAN DIFFERENCE FROM NORMAL STANDARD GRAMS		MEAN DIFFERENCE (PER CENT) IN FOOD FOR SERIES
				Grams per rat per day	Per cent	
		weeks				
Acetic acid, 0.01 per cent	56	11	−2.7	−1.6	−15	−5.5
Acetic acid, 0.01 per cent	107	9	+0.04	+0.45	+4	
Acetic acid, 0.1 per cent	63	15	+0.21	+0.2	+1	+1.0
Acetic acid, 0.25 per cent (after 10 weeks of 0.01 per cent)	5695	15	−1.06	−0.8	−7	+7.0
Acetic acid 0.5 per cent (after 16 weeks of 0.1 per cent)	6295	9	−2.6	−3.8	−27	−27.0
Formic acid, 0.01 per cent	57	11	−1.3	−0.4	−4	−1.0
Formic acid, 0.01 per cent	108	14	−0.63	+0.2	+2	
Formic acid, 0.1 per cent	64	15	−0.13	+1.0	+8	+8.0
Formic acid, 0.25 per cent (after 12 weeks of 0.01 per cent)	5796	15	+0.6	+0.6	+6	+6.0
Formic acid, 0.5 per cent (after 17 weeks of 0.1 per cent)	6496	9	−0.66	−3.8	−29	−29.0

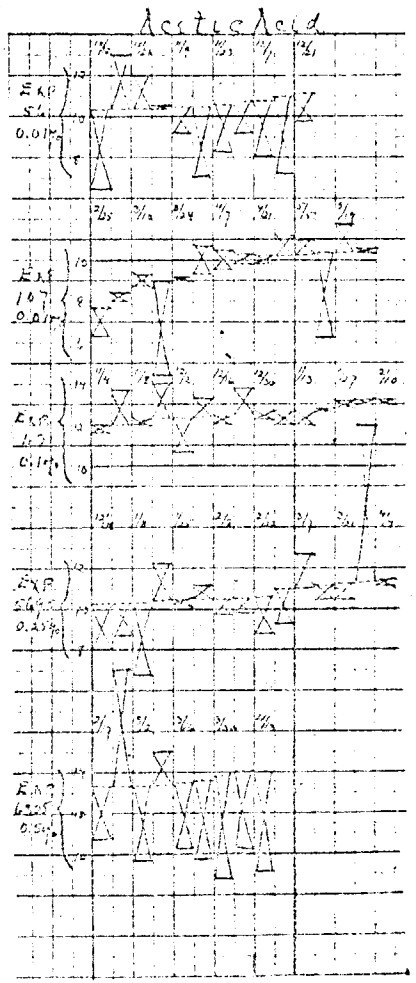

FIG. 4. ACETIC ACID ON FOOD CONSUMPTION

The numbers to the left represent grams of feed consumed daily per rat. The numbers above each experiment are the dates of the observations. The dotted horizontal lines represent the standard food consumption; and the solid horizontal lines, joined to the dotted lines by crossed lines represent the actual food consumption.

food averages lies between −0.01 and +1.4 grams per rat per day, or −0.08 to +19 per cent.

The experiments with 0.1 per cent and 0.25 per cent acetic and formic acids arrange themselves in close proximity to both extremes; that is, the food consumption is normal; if anything, formic acid seems to stimulate appetite somewhat; but this is

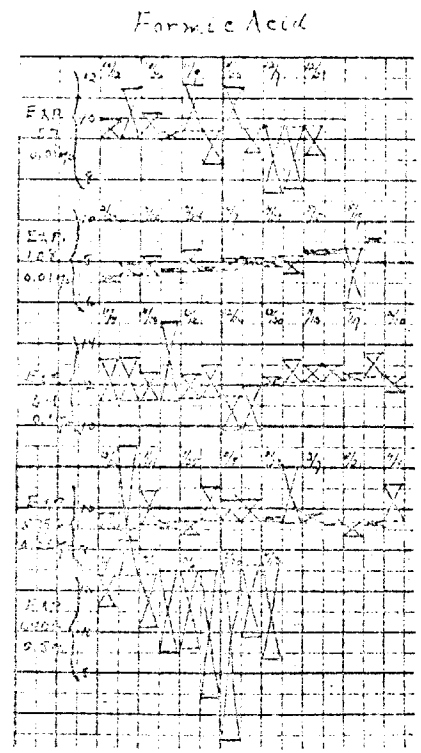

FIG. 5. FORMIC ACID ON FOOD CONSUMPTION

doubtless an accidental coincidence. With the 0.01 per cent acids, one experiment for each acid gave normal results, and the other a deficit; doubtless accidental, as explained under growth.

With the 0.5 per cent acids, there is serious interference with appetite; the food-consumption being reduced to 27 per cent for each acid.

The food and growth curves are in general very closely parallel. It is therefore impossible to say whether the failure of growth is the consequence of the failure of appetite, or vice versa.

MORTALITY

The data are shown in table 5. Briefly, no fatality occurred with either acid for concentrations of 0.5 per cent, equivalent to 0.36 cc. per kilogram of rat per day, for nine weeks, following after sixteen weeks of 0.1 per cent. In view of this, the occasional deaths from lower concentrations must be considered as accidental coincidences.

TABLE 5

Mortality

DRUG AND CONCENTRATION	DOSAGE	EXPERIMENT NUMBER	NUMBER OF ANIMALS	WEEKS OF FATALITIES	TOTAL DURATION	PER CENT OF FATALITIES
					weeks	
Acetic acid, 0.01 per cent	8.2	56	6	8	11	16
Acetic acid, 0.01 per cent	10.9	107	3	0	9	0
Acetic acid, 0.1 per cent	81.0	63	6	0	15	0
Acetic acid, 0.25 per cent (after 10 weeks of 0.01 per cent)	210.0	5695	4	2, 4, 11	15	75
Acetic acid, 0.5 per cent (after 16 weeks of 0.1 per cent)	360.0	6305	3	0	9	0
Formic acid, 0.01 per cent	8.2	57	6	11	11	16
Formic acid, 0.01 per cent	10.25	108	3	0	14	0
Formic acid, 0.1 per cent	90.0	64	6	0	15	0
Formic acid, 0.25 per cent (after 12 weeks of 0.01 per cent)	160.0	5796	4	2	15	25
Formic acid, 0.5 per cent (after 17 weeks of 0.1 per cent)	360.0	6406	3	0	9	0

CONCLUSIONS

Acetic and formic acids behave approximately quantitatively alike, when added to the drinking water of rats, in concentrations up to 0.5 per cent and daily doses up to 0.36 cc. of absolute acid per kilogram of body weight, for two to four months; this being the sole source of fluid for the animals.

Concentrations of 0.01 per cent to 0.25 per cent, corresponding to daily dosage of 0.2 cc. of acid per kilogram of body weight, produced no effect on growth, appetite, or consumption of fluid. They are therefore quite harmless.

The diuresis described in the literature for formates and acetates must be due to the sodium, potassium or lithium, and not to the formic radicle.

With concentrations of 0.5 per cent and daily dosage of 0.36 cc. of absolute acid per kilogram of rat, the appetite and growth but not the fluid consumption are materially, immediately and progressively diminished with both acids. This is evidently due to their acidity; but the experiments do not throw any light as to whether this is due to local action on digestion, or whether to some more profound disturbance of the acid-base equilibrium.

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A transepithelial pump for weak electrolytes

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STEVENS, C. E., A. DOBSON, AND J. H. MAMMANO. *A transepithelial pump for weak electrolytes.* Am. J. Physiol. 216(4): 983–987. 1969.—The isolated rumen epithelium was shown to transport three weak organic acids; acetate, 3,3-dimethylbutyrate, and 5,5-dimethyloxazolidine-2,4-dione in the direction of blood to lumen, and two weak organic bases: morphine and strychnine, from lumen to blood. There was a net transport of each of these against an electrochemical difference and in the absence of a pH gradient between the bathing solutions. A model is presented which could explain these results on the basis of an electrochemical gradient of hydrogen ion between a tissue compartment and the bathing solutions plus a difference in the permeability of the membranes separating the tissue compartment from the opposing bathing solutions.

organic acid transport; organic base transport; epithelial transport; gastrointestinal absorption; ruminant forestomach

THE ISOLATED EPITHELIUM of the ruminant forestomach transports acetate in the direction of blood to lumen, against an electrochemical potential difference and in the absence of a pH difference between opposing bathing solutions (13). Since, in addition, the net transport of acetate was also in a direction opposite to that of net water movement, one of the tissue membranes may be capable of actively transporting acetate.

However, earlier studies indicated that the tissue uptake of acetate from the lumen bath was much more rapid during the first 0.5 hr of the experiment (11) indicating that the rate-limiting barrier to passive diffusion is [illegible] at the lumen surface. Moreover, the transport of acetate from lumen to blood is not affected by an electrical potential (12) indicating a tissue barrier to passive diffusion of the anion. In vivo experiments also indicate that a large percentage of the acetate leaves the rumen [illegible] undissociated form even from solutions of neutral [illegible]. These results suggested the alternative hypothesis [illegible] a tissue compartment is separated from the two bathing solutions by two membranes which differ in their relative permeability to the dissociated and undissociated forms of acetate. Under these conditions a hydrogen ion gradient, maintained between the tissue compartment and the bathing solutions, could provide the energy for the apparent active transport of acetate. This hypothesis was further tested in the following study by determining whether the transport system was specific for acetate or applied in a predictable manner to other weak organic acids and bases.

METHODS

The general experimental procedure for studying transport has been previously described (11). Tissues were preincubated in the bathing solution subsequently used in the experiment and placed between the two bathing chambers. Identical solutions were added to both sides of the tissue, except where specifically noted below, and the transepithelial electrical potential was short circuited throughout the experiment. Solutions were either buffered with tris(hydroxymethyl)methylglycine (Tricine) and gassed with 100% O_2, or buffered with bicarbonate and gassed with 95% O_2–5% CO_2. At the end of the 2.5-hr experiments the opposing solutions, in 23 observations, differed by 0.00 ± 0.01 (SE) pH units and pH varied little when tested at intervals during this period, so that transport was studied in the absence of either an electrochemical or pH gradient between these solutions. The net change of organic acid or base in each bathing solution was determined by measuring its concentration and either using creatinine as a volume marker or rinsing the chamber with an equal volume of normal Ringer solution. The two procedures were found to give equivalent results if the rinsing process was brief to prevent extraction of the organic acid or base from the tissue. Rinse solutions were removed again in less than 1 min.

5,5-Dimethyloxazolidine-2,4-dione (DMO) was [illegible] to measure the pH of the epithelial tissue contents [illegible]. Isotopically tagged inulin was used to measure [illegible] fluid volume. Although the DMO method [illegible] values for intracellular pH than methods [illegible] electrode (2) and it is not known whether [illegible] trates the intercellular space of the epithelial layer [illegible] it was felt that this method would give a maximal [illegible] value for the epithelial tissue compartments.

Selection of organic acids and bases. Two criteria were applied in selecting the experimental procedures and the compounds tested for transport. The first was that there should be no toxic effect on the tissue as evidence by changes in its short-circuit current or resistance. For example, attempts to reverse the direction of net acetate

transport by lowering the pH of the two bathing solutions showed that transport diminished with a decrease in pH until at pH 5.9 acetate was absorbed from both solutions. However, this was also accompanied by a marked decrease in short-circuit current indicating possible tissue damage, so this approach was not further pursued. The ranges of tissue short-circuit current and resistance in the following experiments were not significantly different from those previously measured during acetate transport (12). The second criterion was that the compounds selected for study must not be metabolized to any great extent by rumen epithelium. Previous studies (11) demonstrated the marked effect of butyrate metabolism on its transport. Therefore 3,3-dimethylbutyrate was tested as an organic acid less vulnerable to metabolism. Table 1 compares the transport of butyrate and 3,3-dimethylbutyrate when tissues were bathed between solutions buffered at pH 7.4 with bicarbonate. It can be seen that although butyrate was absorbed at approximately the same rate from both bathing solutions, 3,3-dimethylbutyrate showed a much slower rate of absorption from the blood side and a net transport into the lumen bath. A comparison of total ketone body levels also demonstrates the marked difference in the metabolism of these two acids. Therefore, 3,3-dimethylbutyrate was chosen as one acid for comparative study. Butyrate, dimethylbutyrate, and acetate concentrations were determined by steam distillation and titration (11).

DMO which is generally accepted as a nontoxic, nonmetabolizable weak organic acid was selected as one acid for comparison. Assuming no metabolism, its concentration was determined by radioisotope measurement of the ^{14}C-labeled compound.

The choice of organic bases was limited by the biological activity of many of the more obvious alkaloids. Caffeine, for example, was found to produce a marked inhibition of short-circuit current even in the lowest concentrations tried. Morphine and strychnine had no noticeable affect on tissue short-circuit current or resistance. In addition, spectrophotometric analysis of control and experimental bathing solutions showed no interference by other substances in the ultraviolet absorption spectrum of these two compounds. This allowed their respective measurement at wave lengths of 297 and 274 mμ without previous ether extraction.

TABLE 1. *Comparison of butyrate and 3,3-dimethylbutyrate transport in bicarbonate-buffered Ringer solution*

Acid Concn of Initial Bath Soln	No. of Tissues	Net Change in Bath Solutions: Lumen Side, μmoles ± SE	Net Change in Bath Solutions: Blood Side, μmoles ± SE	Total Ketone Bodies, μmoles ± SE
10 mM butyrate	5	−36.9±6.9	−24.2±6.5	63.1±4.8
10 mM 3,3-dimethylbutyrate	10	+3.9±1.0	−5.6±1.0	2.0±0.5

In each experiment the same solution was added to both sides of the tissue. Results represent net change in the amount of organic acid expected in the bathing solution during a 2.5-hr period in absence of electrical and pH differences between bathing solutions. Last column refers to total amount of ketone bodies recovered from the solutions and tissue at end of the experiment. The area of tissue diaphragm separating the chambers in this and subsequent experiments was 7.1 cm².

The ability of rumen epithelium to concentrate acetate in the lumen bath was studied in a series of experiments varying the concentration gradient of acetate across the tissue. After preincubation in 5 mM acetate-Ringer solution the tissue was divided into a pair of samples. To one tissue of each pair a large volume of Ringer solution was added to the lumen bath. Different concentrations of acetate were tested in this bath to find one which maintained no net exchange of acetate between the tissue and a 5 mM acetate-Ringer solution bathing its blood side. This was reached at lumen concentrations of about 15 mM acetate. With the other tissue of the pair large volumes of varying acetate concentration were added to the blood side and it was found that a concentration of 7.5 mM acetate gave no net exchange between tissue and a 5 mM acetate-Ringer solution on the lumen side. Assuming the two tissues metabolized acetate at approximately the same rate, in the absence of metabolism acetate transport would have reached a steady state at a concentration ratio of about 15:7.5 or 2:1 between lumen and blood.

The DMO method indicated an intracepithelial pH of 7.1 ± 0.1 (SE) in tissues bathed in 40 mM acetate-Ringer solution buffered at pH 7.4. Inulin space represented about 35% of the tissue volume which may have represented only the interstitial volume of the subepithelial connective tissue. Although this pH difference would be sufficient to explain the 2:1 concentration ratio of acetate, the subsequent demonstration that the tissue transports

TABLE 2. *Transport of weak acids and bases by rumen epithelium in Tricine-buffered Ringer solution*

Acid or Base Concn of Initial Bath Soln	No. of Tissues	Net Change in Bath Solutions: Lumen Side, μmoles ± SE	Net Change in Bath Solutions: Blood Side, μmoles ± SE
Acid			
10 mM acetate	6	+10.3±1.0	−15.1±0.7
10 mM 3,3-dimethylbutyrate	6	+9.1±1.2	−10.6±[illegible]
10 mM DMO	7	+6.8±1.3	−5.7±0.[illegible]
Base			
2.0 mM morphine	4	−1.6±0.3	+1.9±0.[illegible]
0.5 mM strychnine	8	−0.8±0.1	+0.9±0.[illegible]

In each experiment the same solution was added to both sides of the tissue. Results represent net change over a 2.5-hr period in absence of electrical and pH differences between opposite bathing solutions. Differences between values for 3,3-dimethylbutyrate transport in Tables 1 and 2 could be attributed to the buffers used since acetate transport is similarly affected by the use of the two buffers (11).

DMO questions a basic premise of the method, i.e., impermeability of compartment membranes to its dissociated form. In addition, the net transport of DMO across the tissue is likely to result in concentration gradients within the epithelium.

Table 2 lists the results of acetate transport previously described (13), along with the results obtained from the present study of 3,3-dimethylbutyrate, DMO, morphine, and strychnine transport between Tricine-buffered solutions. In each series of experiments the identical solution of Ringer containing the organic acid or base was added to both sides of the tissue and transport was studied in the absence of electrochemical or pH gradients over the 2.5-hr experimental period. It can be seen that each of the acids and bases showed a net transport across the epithelium with the organic acids moving in the direction of blood to lumen and the organic bases in the direction of lumen to blood.

DISCUSSION

The observed transport of chemically dissimilar organic acids and bases against their respective electrochemical potential gradients suggests a general mechanism for their transport. To account for the net movements of weak acids and bases in opposite directions a three-compartment system is considered, consisting of a membrane P separating *solution 1* from *solution 2* and a membrane Q separating *solution 2* from *solution 3*. It is assumed that a steady concentration with time has been reached of the components under consideration and that concentration gradients are zero except within the membranes. Furthermore, it is assumed that both the pH and the electrical potential of *solutions 1* and *3* are the same. An expression for the concentration ratio of the undissociated form of a weak acid across a single membrane was introduced and described by Roos (9). This was derived by considering the diffusion of charged and uncharged forms of a weak acid across a membrane, and applying the condition for no net transfer, when the flux of uncharged form in one direction is equal to the flux of charged form in the opposite direction. The expression for the flux of charged form was derived by employing the constant field assumption of Goldman (5). Applying the equation to membrane P, the ratio of concentrations of the uncharged form P_u, is given by:

$$P_u = \frac{c_2}{c_1} = \frac{p + \dfrac{p^- K_c FV}{H_2 RT} \cdot \dfrac{1}{1 - \exp - \dfrac{FV}{RT}}}{p + \dfrac{p^- K_c FV}{H_2 RT} \cdot \dfrac{\exp - \dfrac{FV}{RT}}{1 - \exp - \dfrac{FV}{RT}}} \qquad (1)$$

where the subscripts 1 and 2 refer to solutions, and [illegible] is used to denote a weak acid; p is the concentration of uncharged form of the weak acid; p and p^- are the permeability coefficients of membrane P to the uncharged and charged forms; H is the hydrogen-ion concentration; V the potential of *solution 2* with respect to *1* and F, R, T, and K_c have their usual connotation.

By rearranging and substituting, this expression can be written:

$$P_u = \frac{r_p^- \alpha + 1}{r_p^- \alpha + \dfrac{a_1}{a_2}} \qquad (2)$$

where r_p^- is the ratio of the relative permeabilities p/p^- and where the electrochemical activity ratio for hydrogen ion:

$$\frac{a_1}{a_2} = \frac{H_1}{H_2 \exp \dfrac{FV}{RT}}$$

and

$$\alpha = \frac{H_1 RT}{K_c FV}\left(1 - \exp - \frac{FV}{RT}\right)$$

An expression analogous to *equation 2* describes the concentration ratio of the uncharged form of a weak acid across membrane Q. Remembering that $H_1 = H_3$ and $a_1 = a_3$ we may write:

$$Q_u = \frac{c_2}{c_3} = \frac{r_q^- \alpha + 1}{r_q^- \alpha + \dfrac{a_1}{a_2}} \qquad (3)$$

The overall concentration ratio of the uncharged form of the weak acid across both membranes, G_u, is given by:

$$G_u = \frac{c_3}{c_1} = \frac{P_u}{Q_u} = \frac{r_p^- \alpha + 1}{r_p^- \alpha + \dfrac{a_1}{a_2}} \cdot \frac{r_q^- \alpha + \dfrac{a_1}{a_2}}{r_q^- \alpha + 1} \qquad (4)$$

Since $H_1 = H_3$, G_u is also the concentration ratio of the charged form of the weak acid. By inspection of *equation 4* it is apparent that two conditions are necessary for the double-membrane system to concentrate a weak acid: there is a structural requirement that the relative permeabilities of the two membranes shall differ, i.e., $r_p^- \neq r_q^-$. Also there is an energy requirement that there shall be a difference of electrochemical activity of hydrogen ion between the inner compartment and the two outer compartments, i.e., $a_1/a_2 \neq 1$. The maintenance of such an electrochemical gradient requires the continuous expenditure of energy since the hydrogen-ion gradient would be dissipated by weak electrolyte movements and not by diffusion of hydrogen ions [illegible].

The expression corresponding to *equation 2* for the distribution of a weak base which is derived similarly to

equation 1 (9) is given by:

$$P_a = \frac{r_p^+ \beta + 1}{r_p^+ \beta + \frac{a_2}{a_1}} \qquad (5)$$

where:

$$\beta = \frac{K_a RT}{H_1 FV} \cdot \frac{\left(1 - \exp - \frac{FV}{RT}\right)}{\exp - \frac{FV}{RT}}$$

This leads to an expression for the overall concentrating ability:

$$G_a = \frac{c_1}{c_2} = \frac{r_p^+ \beta + 1 \quad r_q^+ \beta + \frac{a_2}{a_1}}{r_p^+ \beta + \frac{a_2}{a_1} \quad r_q^+ \beta + 1} \qquad (6)$$

Comparing *equations 4* and *6* it is apparent that the system will concentrate weak bases on one side and weak acids on the other if the same membrane which is relatively more permeable to the anionic form of an acid is also relatively more permeable to the cationic form of the weak base. If the concentration of weak electrolyte on both sides is initially the same, net transfer of a base will be in a direction opposite to an acid. The energy for this would be derived from the hydrogen-ion activity ratio.

The properties of the model can conveniently be considered further by examining *equation 2*. For a given electrochemical activity, a_1/a_2, different values of P_a are generated by assigning values to $r_p^+ \alpha$ (Fig. 1). For a given double-membrane system, a_1/a_2 and α are the same for both P_a and Q_a so they will both lie on the same line. Thus the effect of varying the permeability properties of the two membranes can be studied by choosing two points on the same line to represent P_a and Q_a, and then calculating G_a from *equation 4*. The maximum concentrating ability is realized by choosing P_a so that it approaches a_2/a_1 and Q_a so that it approaches 1, when G_a approaches a_2/a_1. Thus the observed twofold concentration of acetate would require at least a twofold hydrogen-ion activity ratio. This could arise if the cellular pH was 0.3 unit below that of the bathing medium if no electrical potential was present. Alternatively, a potential of −19 mv with no pH difference would produce this effect with the permeability characteristic of the membranes P and Q reversed. This minimal hydrogen-ion activity ratio would, however, require the relative permeabilities r_q^+/r_p^+ to exceed 10^6. However, much less disparity in membrane permeability would be necessary with a greater driving force. For instance, if $a_1/a_2 = 1/3$, the twofold concentration of weak acid could be given by choosing points A and B (Fig. 1) for P_a and Q_a. This would require only a 20-fold ratio of relative permeabilities.

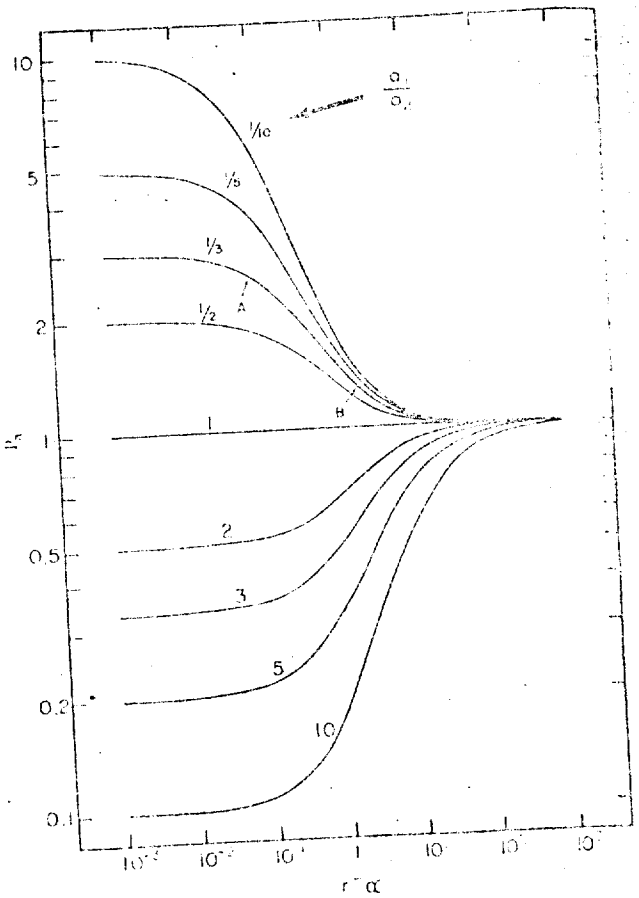

FIG. 1. Concentration ratio (P_a) of undissociated acid across a single membrane as a function of membrane properties. Concentration ratio is plotted against a function ($r^+\alpha$) of the ratio of permeabilities in the dissociated and undissociated form. Each line represents a given electrochemical activity ratio for hydrogen ions (a_1/a_2) across the membrane. For a given double-membrane system, points for both membranes lie on the same line. Points A and B represent a hypothetical double-membrane system giving a twofold concentration of weak acid (see text). The same graph would apply to weak bases simply by substituting P_b for P_a, $r^+\beta$ for $r^+\alpha$, and referring the numbers on each line to a_2/a_1 ($r^+\beta$ for acids, $r^+\alpha$ for bases).

A further restriction on the system is apparent if the relative permeabilities of charged and uncharged forms are constrained within a narrow range, for instance if both r_p and r_q lie between 10 and 10^5, the concentrating ability of the system would be manifest only with a weak acid of $10^{-2} > K_a > 10^{-5}$ or a weak base of $10^{-6} > K_a > 10^{-12}$.

The epithelium of the rumen, like the skin of the frog, is a stratified squamous epithelium which actively transports Na. Ussing and Windhager (11) have presented evidence for the location of two barriers in frog skin which differ markedly in their permeability to Na. One barrier, permeable to Na and impermeable to K, they located just underneath the stratum corneum, separating the underlying epithelial cell contents from the [illegible] of the skin. Another was the membrane separating [illegible]

cell contents from the intercellular space. In the present study, a limited number of experiments using frog skin indicated that, unlike rumen epithelium, it does not transport acetate, DMO, or morphine against an electrochemical gradient. However, the permeability of the lumen-facing membrane of rumen epithelium to K (4) also suggests that its barrier within or near the stratum corneum is more permeable to substances other than Na. The rumen epithelial cell thus could provide the tissue compartment described in the present model or, if the stratum corneum and basement membrane present sufficient diffusion barriers, the intercellular space would provide an alternative tissue compartment.

In the intestine and colon, there is also evidence that weak organic electrolytes do not distribute between lumen and blood strictly according to the conventional ion-trapping formulas. Quinine equilibrates across the colon wall as if the pH of the lumen contents were 0.3 unit below that measured (10), whereas other organic acids and bases equilibrate as if the pH within the intestine were 0.5–1.3 units lower than that measured in lumen contents (7). This has been explained by postulating a thin layer at the lumen surface of the epithelium, maintained at a pH lower than that of the bulk intestinal solution (7). The double-membrane system proposed for the rumen bears a resemblance to this model if the whole epithelium is considered as one membrane, and free diffusion in solution as the other. The double-membrane system does, however, direct attention to the electrochemical rather than the chemical activity of hydrogen ion as the source of energy. In the intestine there is considerable evidence for a structural and permeability difference between the lumen-facing membranes of intestinal cells and the membranes forming their lateral and basal border (6). This difference in permeability is also incorporated into Curran's (3) double-membrane model for transport of water across the rat ileum.

The structural requirements for the proposed double-membrane model thus appear to be reasonable for both ruminal and intestinal epithelia. Given suitable electrodes, it should be possible to test whether the energy requirements are satisfied.

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Formaldehyde-Induced Mutations in *Drosophila melanogaster* in Dependence of the Presence of Acids

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Summary. The mutagenic activity of various combinations of formaldehyde, formic acid, acetic acid and hydrochloric acid was investigated by a sex-linked lethal test. All combinations were mutagenic and showed a mutating pattern from which it is concluded that in feeding experiments spermatocytes I are especially sensitive to the pairs of chemicals tested. In vapour experiments all germ cell stages were found to be susceptible.

The presence of volatile acids was found to be necessary for the mutagenic activity of formaldehyde in the vapour state. Mutagenic effects were also observed in larval feeding experiments, in which only these acids were added to the medium. Experiments with stabilized pH at 7.5 did not show a significant mutagenic effect of formaldehyde.

It is postulated that the tested agents are catalase inhibitors, which promote the formation of peroxides or free radicals which interfere with DNA replication, thus producing mutations.

Introduction

The sensitivity of *Drosophila* germ cells to the mutagenic action of formaldehyde was described by RAPOPORT (1946) and confirmed by AUERBACH (1949, 1951, 1953, 1957), AUERBACH and MOSER (1953), KAPLAN (1948) and several other investigators (ALDERSON, 1960a, 1960b, 1961, 1964; KRISHNA, 1961; MOSER and AUERBACH, 1961; SOBELS, 1959, 1958, 1962, 1964).

As briefly published earlier (STURM-TIGCHELAAR, 1964) we found that any mutagenic activity of formaldehyde depends on pH values below 7. Detailed experiments which include also the effect of impurities of commercial formaline are presented which support the earlier findings, and give information on the possible mechanisms of mutation induction.

Material and Methods

A standard strain of *Drosophila melanogaster*, Oregon-R (inbred for about 10 years) was used. This strain has a spontaneous mutation rate of 0.15% sex-linked lethals in males.

For *treatments with the vapour phase* of an agent, males were exposed for 24 hrs in specially designed bottles. The flies themselves were kept in small glass tubes (5 : 2.5 cm), which were closed with gauze on the lower end and with cotton wool on the upper end (see figure 1). Samples of each of the agents to be tested (5 ml) were applied to the cotton wool in the bottom of the bottle (volume: 125 ml).

During *larval feeding experiments*, we used a nutrient medium consisting of 12 g of agar, 35 g of milled brewers yeast, 150 g of concentrated 100 g of sucrose per 1000 ml distilled water. This medium was autoclaved, cooled, and supplemented with the chemicals to be tested. These were formaldehyde, formic acid, acetic acid and hydrochloric acid. The pH of the media varied from 4.2 to 5.6 depending on the added reagent. A 37% solution of formaldehyde of pH 7 was obtained by neutralization with 5 N sodium hydroxide. To distinguish the mutagenic effect of commercial formaline (which is known to contain formic acid), it will be referred to as neutral "n"-formaldehyde, and the commercial formaldehyde as acid "a"-formaldehyde. Females were allowed to lay eggs for 24 to 48 hrs on the prepared medium, and were then discarded. F1 flies, which spent their whole larval life on the medium, were collected 24 hrs after emergence (BIRD, 1962) and mated individually, to two of 5 virgins.

For *sex-linked lethals* we employed the MULLER technique (DEMEREC, 1937; STURTEVANT and BRIDGES, 1931). About 50 treated males were individually crossed with one or two virgins of the M-5 strain (also inbred for about 10 years). Every third day the males were transferred to two fresh virgins in order to produce three series of broods.

All experiments were performed at a temperature of 24°C (± 1°C), 60—80% of the flies survive treatment with the tested agents.

In order to test the results statistically, we used the χ^2-test, the rank correlation method of KENDALL (1948), or a special test for mutation frequency of STEVENS (1942).

1. *Vapour Experiments*

The data obtained from vapour experiments are summarized in Table 1. When males were treated 24 hrs after emergence with "a"-formaldehyde, formic acid, acetic acid, hydrochloric acid, or the combination of "a" formaldehyde and the acids, all experiments produced mutations. The data were found to be significantly different ($p \leq 0.001$) from the spontaneous mutation rate of 0.15%.

For each chemical tested, no significant deviation in mutation frequency for sex-linked lethals could be found between the results obtained with each of the three broods (STEVENS, 1942). This result allows us to consider the mutation frequencies for the total of the three broods. If we compare these data, we find significant differences ($p \leq 0.05$) when comparing "a"-formaldehyde with acetic acid; "a"-formaldehyde with hydrochloric acid; "a"-formaldehyde with "a"-formaldehyde + formic acid; and "a"-formaldehyde with "n"-formaldehyde + hydrochloric

Table 1. *Vapour experiments*

treatment		brood							
substance	concentration	1st		2nd		3rd		total	
	%	n[1]	%[2]	n	%	n	%	n	%
nF	7.0	—	—	—	—	—	—	3803	0.16
aF	7.0	436	0.69	712	1.97	423	1.18	1571	1.40
FA	0.1	1068	1.12	1130	1.42	850	1.41	3048	1.31
AA	0.1	1703	0.82	1359	0.66	1270	1.10	4332	0.85
HA	0.01	1017	0.67	977	0.61	396	0.50	2420	0.62
nF + FA	7.0 0.1	1491	0.80	2198	0.82	1809	0.99	5498	0.87
nF + AA	7.0 0.1	786	1.02	1001	1.29	1020	1.18	2810	1.17
nF + HA	7.0 0.01	1263	1.03	1466	0.68	852	0.70	3680	0.79
mutation control		—	—	—	—	—	—	2584	0.15

[1] number of chromosomes tested. — [2] percentage of sex-linked lethals found.

abbreviations:

aF = acid formaldehyde
nF = neutral formaldehyde
FA = formic acid
AA = acetic acid
HA = hydrochloric acid.

All mutation frequencies are significant at the $p < 0.001$ level when compared with the non-treated control.

ric acid. However, no significant differences were found in the comparison of "a"-formaldehyde with "a"-formaldehyde + acetic acid; "a"-formaldehyde with formic acid; and acetic acid with hydrochloric acid. It may be concluded that all germ cell stages can be influenced by "a"-formaldehyde and all the tested acids. It has been shown already in earlier investigations (STUMM-TEGETHOFF, 1964) that "n"-formaldehyde causes no mutation in vapour experiments.

2. *Larval Feeding Experiments*

It could be argued that the acid vapour treatment may interfere with the normal ventilation of the tracheal system. Therefore experiments were carried out with the mutagenic substances in the feeding substrate. As seen from table 2 all tested substances have a significant increased mutagenic effect compared with the control.

In contrast to the results of the vapour experiments the highest mutation rate of sex-linked lethals in most of the larval feeding experiments were found in the second brood. The significance of these results was determined by the rank correlation method. With 0.05% "n"-formaldehyde and 0.05% "n"-formaldehyde + formic acid, however, the highest frequency of mutation was found in the first brood. Within individual experiments there were no significant differences between the broods, as shown by the test of STEVENS (1942), except in the cases of 0.05% "n"-formaldehyde and 0.05% "n"-formaldehyde + formic acid. Therefore, totals were compared in the vapour experiments, regarding no

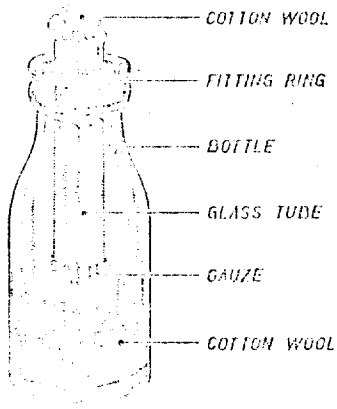

Fig. 1. Bottle for vapour experiments with *Drosophila*

differences between the tested agents except for acetic acid and hydrochloric acid, which were less effective ($p < 0.05$).

The finding that "n"-formaldehyde is as mutagenic as "a"-formaldehyde for larvae, contrasts with the results of the vapour experiments. The pH of "n"-formaldehyde is 7. An explanation could be given by the following experiments.

When hatched larvae were transferred every day to fresh nutrient medium which was adjusted to pH 7.5 with glycine-NaOH buffer (table 3), no mutation was found in the second brood for 0.025% formaldehyde, and one mutation (0.27%) was found for 0.05% formaldehyde.

In another experiment with 0.1% formic acid in the food, but stabilized on pH 7.5 a mutation rate

Table 2. *Larval feeding experiments A*

treatment		Brood							
substance	concentration	1st		2nd		3rd		total	
	%	n[1]	%[2]	n	%	n	%	n	%
nF	0.025	910	0.77	717	1.40	737	0.68	2364	0.93
nF	0.05	1266	1.26	1503	0.33	1601	0.31	4370	0.59
aF	0.025	1287	0.70	1213	0.91	1089	0.73	3589	0.78
aF	0.05	754	1.06	802	1.75	901	0.78	2457	1.18
FA	0.1	786	1.15	522	1.31	571	0.68	1879	1.11
AA	0.1	981	0.61	1007	0.60	1157	0.43	3145	0.51
HA	0.01	757	0.40	668	0.75	800	0.50	2225	0.55
nF + FA	0.025 0.1	839	0.95	857	1.17	587	1.19	2283	1.09
nF + FA	0.05 0.1	981	1.33	1352	0.96	1574	0.76	3907	0.97
nF + AA	0.025 0.1	716	0.98	782	1.28	652	0.77	2150	1.02
nF + AA	0.05 0.1	770	0.91	840	1.79	1092	1.10	2702	1.25
nF + HA	0.025 0.01	478	1.05	591	1.18	548	0.91	1620	1.05
nF + HA	0.05 0.01	446	2.02	514	1.95	447	1.12	1407	1.71
Mutation control	—	—	—	—	—	—	—	2584	0.45

[1] number of chromosomes tested. — [2] percentage of sex-linked lethals found.

Abbreviations:
nF = acid formaldehyde
nF = neutral formaldehyde
FA = formic acid
AA = acetic acid
HA = hydrochloric acid.

All mutation frequencies are significant at the $p < 0.001$ level when compared with the non-treated control.

Table 3. *Larval feeding experiments B*

substance	concentration	pH of the medium	tested chromosomes	mutations found	mutation frequency
nF	0.025%	7.5	398	0	0.00%
nF	0.05%	7.5	366	1	0.27%
FA	0.1%	7.5	544	2	0.38%
Mutation control	—	7.0	2584	4	0.15%

For these experiments a special prepared medium of pH = 7 was used (see "Materials and Methods"). Only the 2nd brood was analyzed. Concentration of the glycine-NaOH buffer: 0.1 M. The mutation frequency shows no significant deviation from the (mutation) control (0.15%) in contrast to the results obtained by testing the same substances in an acid medium.

of 0.38% was observed in the second brood. These data are not significantly different from the spontaneous mutation frequency.

Comparing the results of the feeding experiments one can conclude that there is a positive correlation between the low pH of the nutrient medium and the mutation frequency over all broods.

Discussion

AUERBACH (1951), KAUFMANN and GAY (1963) and EHRSEN (1964) proved that during larval development spermatogenesis stops with spermatocytes I. Therefore, only this type of germ cells (plus spermatogonia) is capable of reacting with mutagenic agents present in the larval food. These findings are in agreement with our results from larval feeding experiments. No clusters of identical lethals from the same male have been found. Therefore, with great probability, only spermatocytes I are influenced (AUERBACH, 1953; SOFUNI, 1954). In comparison with the controls, all broods showed significant higher mutation frequency.

The highest mutation rate occurred in the larval feeding experiments (table 2) in the second brood, which is not in accordance with the data from AUERBACH and MOHR (1953), who found the highest

mutation frequency in the first brood. This is perhaps due to a difference in sensitivity of our standard strain and to the fact that our larvae remained their whole larval lifetime on the treated medium (older larvae incorporate more of the substances than younger ones). All these explanations assume that the males mate only once with the M-5 females within one brood, as suggested by MANNING (1962).

The fact that AUERBACH (1949) did not succeed in mutation induction with formaldehyde vapour is possibly due to a higher concentration employed, which did not allow a longer time of treatment than 2 hrs.

During the larval feeding experiments, it was observed that addition of neutral formaldehyde to the nutrient medium caused a pH decrease, thus producing a mutagenic effect similar to that obtained with "a" formaldehyde. We suggested earlier (STUMM-TEGETHOFF, 1964) that a part of the formaldehyde is oxidized to formic acid, which is also mutagenic.

In the larval feeding experiments, and in some of the vapour experiments, the most sensitive germ cell stages are the spermatocytes I. Probably, DNA replication in these cells is disturbed by the agents (NAFF and AUERBACH, 1964).

SCHILLS (1954, 1958, 1963) suggested that the formation of peroxides was the cause of mutations, a result which is confirmed by other investigators for lower organisms (DICKEY, CLELAND and LOTZ, 1949; JENSEN, KIRK, KOLMARK and WESTERGAARD, 1951). Normally, *Drosophila* larvae contain catalase of high activity. Inhibition of catalase by acids or acidic formaldehyde solutions must lead to an accumulation of mutagenic peroxides. If acids and combinations of formaldehyde with these acids are used in vitro (STUMM-TEGETHOFF and VAN DER LOO, 1969), the resulting catalase inhibition shows a good correlation to the mean mutation frequency obtained in vivo. These findings suggest that, as a consequence of catalase inhibition, peroxides and free radicals are responsible for the mutation. In this regard, BUTLER and COXWOY (1953) proved that OH-radicals are able to break the connection between sugar and phosphate or sugar and base in DNA, and that they are able to oxidize the sugar or the base of nucleosides. In addition, FREESE (1961) found in T4phages that the formation of transversions or transitions by depurinating agents is dependent on a low pH, and can be imitated by a buffer of the same low pH. There may be some relationship between these reports and our results. The absence of a mutagenic effect in the experiments with a buffered medium of pH 7.5 can also be due to the formation of a glycine-formaldehyde complex.

With regard to the larval feeding experiments and the results obtained from the 3rd brood of the vapour experiments, formaldehyde and/or acids may inhibit catalase, as a consequence peroxides and free radicals accumulate; by this process DNA synthesis is disturbed, which leads to mutation. Mutations occurring in the 1st and 2nd broods of the vapour experiments may occur as a consequence of a disturbance of chromosome segregation during meiosis, thus producing repeats and deletions. Other theories of the mutagenic activity of formaldehyde are, however, also compatible with our findings (ALDERSON, 1960a, 1960b, 1961, 1964, 1967).

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Zusammenfassung

Mit Hilfe des Muller 5-Tests wird bei *Drosophila melanogaster* die mutagene Aktivität von Formaldehyd, Ameisensäure, Essigsäure und Salzsäure allein und in verschiedenen Kombinationen von Formaldehyd mit den Säuren geprüft. Alle Kombinationen sind mutagen. Da keine Mutagenität mehr nachweisbar ist, wenn die Fliegen mit Formaldehyd mit einem pH ≥ 7 behandelt werden, ist anzunehmen, daß die mutagene Wirkung von Formaldehyd von einem sauren Milieu abhängig ist. Die untersuchten Substanzen werden den Tieren entweder in der Gasphase oder durch Beimischung zum Nährboden verabreicht. Im ersten Fall werden alle Keimzellstadien beeinflußt, während die Applikation im Nährboden nur die Spermatocyten I angreift. Der Wirkungsmechanismus der untersuchten Stoffe wird als eine Katalase-Hemmung gedeutet, die zur Bildung von u. a. freien Radikalen und damit — über Störung der DNS-Synthese — zum Entstehen von Mutationen führt.

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BISULFITE MUTAGENESIS IN BACTERIOPHAGE T4

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BISULFITE is employed as a preservative in foods and pharmaceuticals. Chemical studies of its interactions with nucleic acid components indicate that it reacts with uracil and cytosine in ways which might be mutagenic (SHAPIRO and WEISRAS 1970; SHAPIRO, COHEN and SERVIS 1970; HAYATSU *et al.* 1970). Two preliminary assays, one in the bacterium *Escherichia coli* (MUKAI, HAWRYLUK and SHAPIRO 1970) and one in bacteriophage lambda (HAYATSU and MIURA 1970), revealed some bisulfite mutagenicity. In both instances, however, a maximum mutagenic response was observed within very short treatment intervals, no further mutagenicity being observed thereafter. The results with *E. coli* showed that only those mutants believed to revert at sites containing guanine:cytosine (G:C) base pairs responded to bisulfite; several mutants probably reverting at adenine:thymine (A:T) base pairs did not respond. Using the T4*rII* system, we have been able to demonstrate a very rigorous specificity of bisulfite mutagenicity for G:C base pairs. (Note, however, that T4 employs the cytosine analogue 5 hydroxymethylcytosine.) Furthermore, both inactivation and mutagenicity are strictly proportional to bisulfite concentration and to time of treatment.

We employed standard methods for the analysis of mutation in T4 (DRAKE 1970). Stocks of various well characterized T4*rII* mutants (DRAKE and MCGUIRE 1967; DRAKE 1963) were first diluted into 0.2% Bacto tryptone + 0.5% NaCl, and then 10-fold either into freshly prepared unbuffered $NaHSO_3$ solutions, or into 0.1 M sodium acetate buffer, at 37°C. The pH of both solutions was 5.0 both before and after incubation. Buffer alone was neither inactivating nor mutagenic, nor did it alter the effects of bisulfite treatment when included in the bisulfite solution. Total viable particles were assayed on BB cells, and r^+ revertants were assayed on KB cells.

Both inactivation and induced reversion of *rIIV55* were studied as functions of bisulfite concentration (Figure 1) and time of treatment (Figure 2). Inactivation exhibits single hit kinetics, with a rate constant of 0.85 M^{-1} hr^{-1} over 0 to 0.9 M and 0 to 4 hr. For this particular *rII* mutant, revertants were induced at the rate of 2.8×10^{-6} M^{-1} hr^{-1}. In stationary phase *E. coli*, on the other hand, using 1 M bisulfite at pH 5.2, a maximum induced mutant frequency was observed within the first treatment interval (0.5 hr), and no further increase occurred over the next 1.5 hr (MUKAI, HAWRYLUK and SHAPIRO 1970). No killing was observed even after 2 hr of treatment. In bacteriophage lambda, a maximum induced mutant frequency was observed by 1.5 hr, and no further increase occurred over the next 1.5 hr (HAYATSU and MIURA 1970). Inactivation was observed in the lambda

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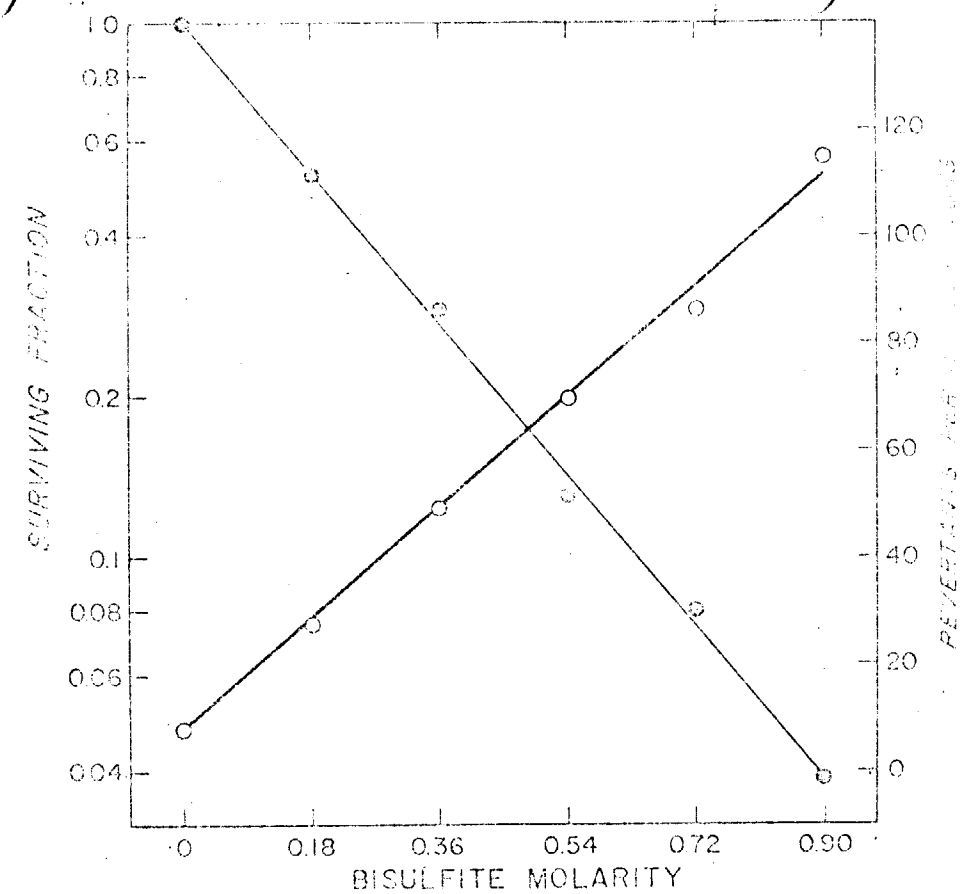

FIGURE 1.—Inactivation (—●—) and reversion (-○-) of T4*rIIV55* as a function of $NaHSO_3$ concentration (treatment interval, 4 hr).

system, the single hit rate constant being 0.68 M^{-1} hr^{-1}, but inactivation in the buffer control (2.3 M NaCl + 1 M sodium phosphate, pH 5.6) occurred nearly (63%) as rapidly (based on a single treatment point), and the similarity of the T4 and lambda inactivation rates is therefore presumably coincidental.

Bisulfite mutagenesis exhibits a very marked allele specificity. When a variety of T4*rII* mutants were treated with 0.9 M bisulfite for four hours (Table 1), only those mutants were reverted by bisulfite which were also reverted by hydroxylamine. These mutants contain G:C base pairs at the reverting site, and can revert by G:C → A:T transitions. Mutants which can revert by A:T → G:C transitions (that is, are reverted by 2 aminopurine but not by hydroxylamine) did not respond significantly to bisulfite, in agreement with the *E. coli* results (MUKAI, HAWRYLUK and SHAPIRO 1970). Frameshift (fs) mutants (reverted by proflavin and not by base analogues) also failed to respond significantly. A number of mu-

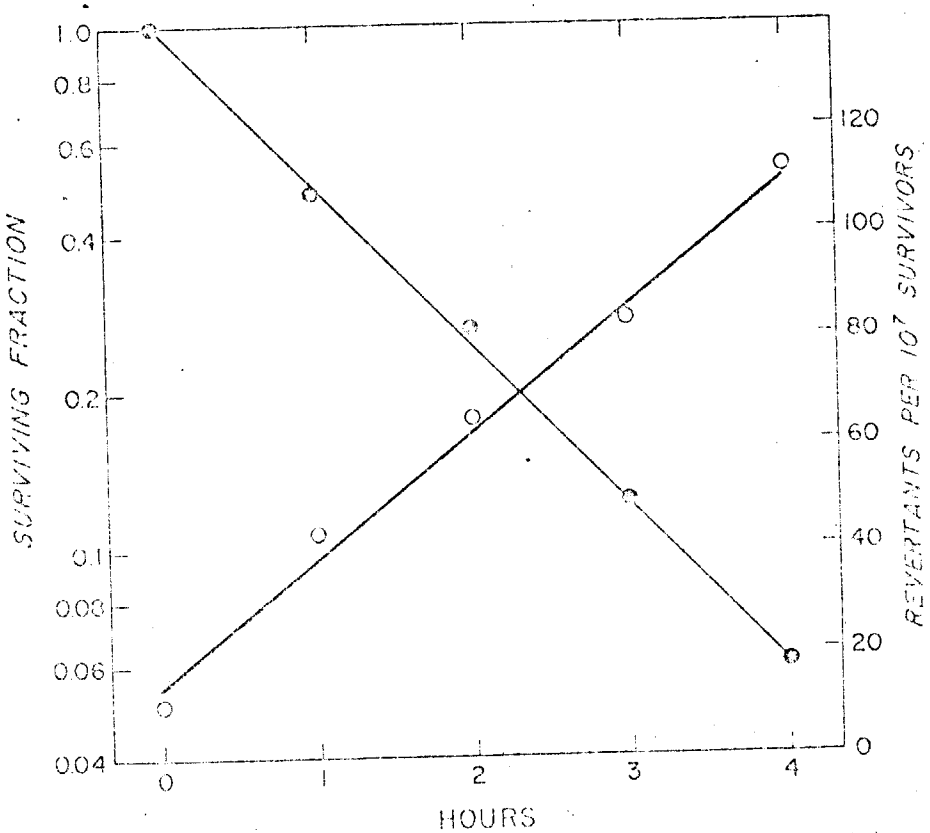

FIGURE 2. Inactivation (●) and reversion (○) of T4rUV55 as a function of time of treatment with 0.9 M $NaHSO_3$.

tants which revert spontaneously, but are not induced to revert by base analogues or by proflavin, also failed to respond to bisulfite. At least one of these mutants, rSM94, probably reverts from a G:C base pair by transversion (tv: purine: pyrimidine → pyrimidine:purine), since it is reverted by cytosine deprivation, which mutates G:C but not A:T base pairs (personal communication from R. BALTZ, E. GREENING and M. MOOGK). Bisulfite therefore not only mutates G:C very specifically, but also probably produces G:C → A:T transitions exclusively.

The contribution of lethal mutations to bisulfite inactivation can be estimated from the data of Table 1. Using the average G:C mutation rate, together with values of 35% for the G:C content and 180,000 for the number of base pairs in T4 DNA, the number of lethal hits per genome turns out to exceed the number of mutational hits by about 13-fold. Assuming that the T4r genes comprise 2% of the genome, we estimate that bisulfite would produce about 0.002 r mutations per lethal hit. By this criterion, bisulfite is a moderately strong mutagen; approximate corresponding values are 0.0002 for ultraviolet irradiation, 0.002 for

TABLE 1

Bisulfite reversion of T4rII mutants

Mutant	Base pair at mutant site	Revertants per 10^7 survivors Control	Treated
rUV7	G:C	1.1	10
rUV48	G:C	0.82	14
rUV55	G:C	8.7	110
rUV256	G:C	4.5	51
rUV363	G:C	2.5	21
rUV16	A:T	0.65	0.87
rUV30	A:T	0.091	0.13
rUV73	A:T	11	11
rUV304	A:T	2.7	4.9
rUV354	A:T	0.82	0.99
rUV6	(fs)	3.1	5.7
rUV28	(fs)	2.4	2.5
rUV58	(fs)	1.3	1.8
rUV113	(fs)	2.4	2.9
rUV353	(fs)	17	20
rSM18	?	2.6	3.2
rSM94	(tv:G:C)	1.1	2.4
rUV34	?	68	71
rUV61	?	1.5	1.8
rUV74	(tv:A:T)	3.4	1.7

nitrous acid, 0.01 for ethyl methanesulfonate, and 0.02 for hydroxylamine (DRAKE 1966). In terms of mutations per mole-hour, bisulfite is about as effective as nitrous acid applied at pH 5.3 (FREESE 1959), but is about 100 times weaker than nitrous acid applied at pH 3.7 (TESSMAN 1962).

Could bisulfite mutagenicity be detected in materials presented for human consumption? Using rUV55 as a tester strain, a sodium bisulfite residue of 0.01 M (about 0.1%) would double the typical background revertant frequency in about 30 hr. with little inactivation. Furthermore, the strictly linear kinetics and concentration dependence of mutation and inactivation observed with rUV55 make this system more suitable than others thus far developed.

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See: Hygiene & Sanitation (Eng. Trans.) 39(1): 122-125, (1969)
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HYGIENIC STANDARDS FOR ACETIC ACID AND ACETIC ANHYDRIDE IN AIR

(Eksperimental'nye dannye k gigienicheskomu normirovaniya soderzhaniya uksusnoi kisloty i uksusnogo angidrida v atmosfernom vozdukhe)

M. F. [illegible]
[illegible] Hygiene of the [illegible] Department of [illegible]

Acetic acid is extensively used as a mordant or solvent in the textile [illegible] in the manufacture of linoleum, acetylcellulose, acetyl[illegible] many other organic syntheses (manufacture of esters, acetates, etc.).

Acetic anhydride is used in the manufacture of acetyl cellulose (for [illegible] cinematographic film), acetobutyrate cellulose, dyes, and [illegible] preparations and in many organic syntheses.

Enterprises manufacturing or consuming acetic acid and anhydride may [illegible] air pollution.

There are no reported data on the effects on man and animals of low [illegible] acetic acid and acetic anhydride (of the order of magnitude [illegible] permissible concentrations in factory air). These [illegible] totally uninvestigated as air pollutants.

We [illegible] experimental investigations to establish the one-time [illegible] concentrations of these substances, using conven[illegible] Acetic acid and anhydride in air were determined [illegible] by the respective methods of Dyuzheva (1960) and [illegible]

[illegible] for the olfactory thresholds and effects on reflexes of [illegible] are listed in the table.

[illegible] to the recommendation that the [illegible] permissible concentrations in air be fixed at 0.1 mg/m³ [illegible] for acetic acid, these levels were [illegible] by the section for air pollution control of the All-Union [illegible]

[illegible] effects of absorption of the two substances and to [illegible] maximum permissible concentrations in [illegible] 105 male albino rats to continuous inhalation [illegible] animals were divided into seven groups, 15 per group. [illegible] concentrations of acetic acid (first, second and third [illegible] fourth, fifth and sixth groups), while the

seventh group provided the controls. The [illegible] 5 mg/m³ acetic acid vapor, [illegible] the maximum permissible concentration [illegible] the second group was exposed to 0.2 mg/m³ [illegible] the average (at the level of the one-time maximum permissible concentration proposed by ourselves), the third group was exposed to [illegible] 0.056 ± 0.010 mg/m³ on the average (this concentration [illegible] lower than that used on the second group by a factor of [illegible]), the fourth group was exposed to acetic anhydride vapor in a concentration [illegible] 0.351 mg/m³ on the average (25 times the one-time maximum permissible concentration), the fifth group was exposed to [illegible] mg/m³ on the average (at the level of the one-time maximum permissible concentration proposed by ourselves), and the sixth group was exposed to a concentration around 70% lower than that used on the fifth group.

Investigation of the chronaxial ratios of antagonistic muscles revealed a distortion of the correct ratio in the first, second, fourth and fifth groups. The chronaxial ratio was inverted in the first and the fourth groups, whereas in the second and the fifth groups the ratios were only reduced. The chronaxial ratios returned to normal in all rats towards the end of the recovery period. Animals of the third and sixth groups exhibited no changes in comparison with the controls (Figure 1).

Threshold concentrations of acetic acid and anhydride for man

Index	Acetic acid		Acetic anhydride	
	concentration, mg/m³			
	liminal	subliminal	liminal	subliminal
Olfactory sensation	[illegible]	0.01	[illegible]	0.41
Ocular sensitivity to light	0.48	[illegible]	[illegible]	[illegible]
Electrical activity of cerebral cortex	[illegible]	0.1	.1	[illegible]

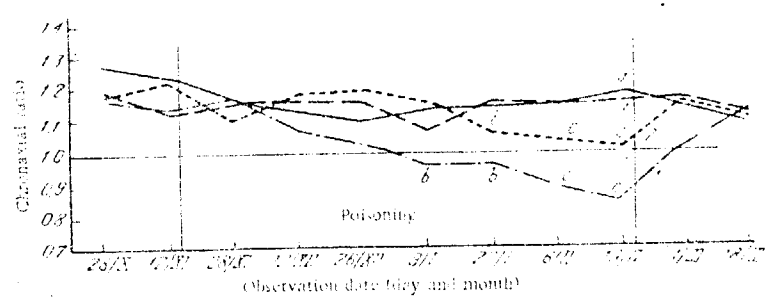

FIGURE 1. Chronaxial ratios of antagonistic muscles in animals poisoned with acetic acid vapor

1 – 1st group (0.07 mg/m³); 2 – 2nd group (0.204 mg/m³); 3 – 3rd group (0.06 mg/m³); 4 – 7th group (controls); a, b, c – [illegible]

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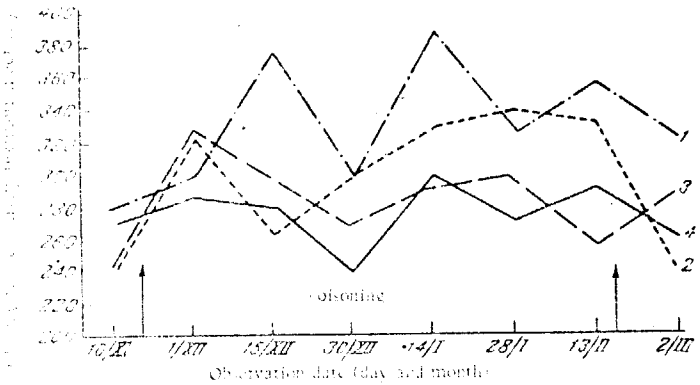
Whol[illegible] cholinesterase activity was determined twice monthly, [illegible] cation of the method proposed by Fleisher and Pope (1954). Cholinesterase activity was raised by acetic acid concentrations of 5 and 0.2 mg/m³ and by acetic anhydride concentrations of 2.5 and 0.1 mg/m³. There were no statistically significant changes in the third and sixth groups (Figure 2).



[illegible] Whole blood cholinesterase activity in rats during long-term poisoning [illegible] anhydride

[illegible] 2 — 5th group (0.10 mg/m³); 3 — 6th group (0.028 [illegible] 4 — [illegible] (controls).

Total proteins and protein fractions in the blood serum were investigated at 25 [illegible] intervals. The fractions were investigated by paper electrophoresis, after which the phoregram was reproduced on photographic paper and the different fractions were weighed. There were no statistically significant changes in total serum proteins. Changes were discovered in the protein fractions of the rats in the first, second, fourth and fifth groups. In the first and fourth groups, there was a decrease in albumens and an increase in alpha, beta and gamma globulins, with a corresponding decrease in the albumen/alpha-globulin, albumen/beta-globulin and albumen/gamma-globulin ratios. The changes in the albumen and beta globulin fractions were still present on the 25th day of the recovery period. Rats of the [illegible] exhibited a slight decrease of albumens and an [illegible] globulins only, with a corresponding decrease of the [illegible]. No such changes were observed in the third [illegible].

[illegible] was determined in the diurnal urine of 5 rats in every [illegible] intervals. In the first and fourth groups, there was a [illegible] decrease 6 weeks after the beginning of poisoning. [illegible] either group, and disappeared two weeks after [illegible] poisoning. Rats in the second, third, fifth and sixth [illegible] exhibit any statistically significant changes in comparison [illegible].

[illegible] function was studied by Quick-Pytel's method, as modified by [illegible]. After a month of poisoning, rats in the first and fourth [illegible] increase of hippuric acid in the urine, followed

by a statistically significant decrease at the end of the [illegible] period, 29.6 – 34 % in comparison with the controls. In some of the animals [illegible] significant changes were detected in rats of the second, third, fifth and sixth groups, in comparison with the controls.

The morphological composition of blood was studied at 14-day intervals. The erythrocyte count and hemoglobin level were within normal limits in all the groups. It should, however, be noted that the rats in the first and fourth groups displayed some tendency towards a decrease of both erythrocytes and hemoglobin, but the differences were not statistically significant. Animals of the first and fourth groups had persistent leukocytosis during the poisoning period. There were no significant changes in the WBC in the second, third, fifth and sixth groups.

In order to detect possible hemolysis, we attempted to determine the resistance of erythrocytes to UV-irradiation, according to the method devised by Mochalina. This method of studying the protohemolytic resistance of erythrocytes is based on the assumption that additional treatment (by UV-irradiation) of the erythrocytes of an animal already exposed to injurious (hemolytic) substances might reveal fine changes that cannot be detected under ordinary conditions. Our data suggest a decrease in the hemolytic resistance of erythrocytes in the first and fourth groups. Rats of the second, third, fifth and sixth groups exhibited some tendency towards a diminished resistance of erythrocytes but the changes were not statistically significant.

At the end of the experiment, determinations were performed of the content of ascorbic acid in the liver, kidneys and adrenals of four rats from each group. In the first and the fourth groups there was a decrease in the vitamin C content of the liver (44.2 %), kidneys (40.1 %) and adrenals (43 %). No statistically significant changes were discovered in the other groups.

Long-term poisoning of albino rats with acetic acid and acetic anhydride vapors for 95 days thus caused changes in several indices in the first, second, fourth and fifth groups, but none in the third and sixth groups.

On the basis of our experimental results, we proposed that the mean diurnal maximum permissible concentrations of acetic acid and anhydride be set at 0.06 and 0.03 mg/m³ respectively. Investigations of the air around a chemical factory manufacturing and consuming 20,000 tons acetic anhydride and 22,000 tons acetic acid per year revealed acetic acid concentrations 2 – 5 times above the one-time maximum permissible concentration at distances of up to 500 m; the concentration of this air pollutant dropped below its one-time maximum permissible concentration only at a distance of 1,000 m. Acetic anhydride concentrations were 2 – 5 times above the one-time maximum permissible concentration within a radius of up to 300 m; they were below the one-time maximum permissible concentration at a distance of 500 m.

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ACETATE AND CHOLESTEROL METABOLISM IN THE HUMAN FOETO-PLACENTAL UNIT AT MIDGESTATION.

I. *Synthesis of cholesterol*

By

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ABSTRACT

As the first part of a series of investigations on acetate and cholesterol metabolism, the conversion of acetate to cholesterol was studied in the various compartments of the midgestation foeto-placental unit in perfusion experiments carried out for 90 min at 35–36° C.

Following their removal at laparotomy two complete foeto-placental units were perfused each with 5.0 mCi of uniformly labelled sodium acetate-^{14}C + 5.0 mCi of cholesterol-7α-^{3}H. The study was completed by the separate perfusion of two isolated midgestation foetuses and two midgestation placentas. The doses administered in each of these four last experiments were 2.5 mCi of ^{14}C-labelled acetate and 2.5 mCi of ^{3}H-labelled cholesterol.

Cholesterol was isolated in a radiochemically homogeneous form from each of the tissues studied.

The cholesterol isolated from the placentas, placental perfusates and from the blood bathing the placenta from the maternal side (»maternal perfusates») contained exclusively ^{3}H-label. On the other hand, the cholesterol isolated from all foetal livers, adrenals, testicles, 3 of 4 residual foetal tissues and 1 out of 4 foetal perfusates also contained significant quantities of ^{14}C-label.

It is concluded, that the midgestation human placenta is not capable of synthesizing cholesterol from acetate, but that the conversion of acetate to cholesterol is a quantitatively significant metabolic pathway in the human foetus at midgestation.

A preliminary report has been presented at the 7th Acta Endocrinologica Congress, Stockholm, June 29 – July 2, 1969 (*Telegdy et al.* 1969).

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The steroid metabolism of the human foeto-placental unit at midgestation has been extensively studied during the past few years (for a review: *Diczfalusy* 1969). Many steps of these steroidogenic processes were established on the basis of *in vivo* experiments.

However, we could not conduct clinical studies to assess the significance of *de novo* steroid synthesis from acetate, because of the possible radiation hazards involved. Recently a method has been developed in this laboratory for the perfusion of the complete foeto-placental unit, following its removal from the uterus (*Lerner & Diczfalusy* 1968). Using this method it became possible to perfuse the foeto-placental unit with sufficiently large amounts of labelled acetate and cholesterol.

The present paper describes studies on cholesterol synthesis from acetate, including perfusions of the midgestation foeto-placental unit, as well as perfusions of isolated placentas and foetuses. Subsequent papers will deal with the synthesis of various steroids from acetate and cholesterol under these experimental conditions.

EXPERIMENTAL

Clinical material. – Two complete foeto-placental units were perfused (Experiments No. 1 and 2) which were obtained following laparotomy in connection with the interruption of gestation. In the other experiments only the foetuses (Experiments No. 3 and 4) or placentas (Experiments No. 5 and 6) were perfused separately following their removal at laparotomy. All foetuses were male ones. Permission for interruption of gestation was granted by the Royal Medical Board of Sweden upon request of the patients under the statute of 1938, as amended in 1946 and 1953. The period of gestation varied between 18 and 20 weeks.

Conditions of perfusion. – These were carried out at 35–36 C with heparinized oxygenated fresh Rh –, 0-blood diluted with 15 % (v/v) saline. The oxygen contained 5 % CO_2. Perfusion time was 90 min in all experiments, except in No. 2 in which it was 83 min. Perfusion of the foeto-placental unit was carried out as described by *Lerner & Diczfalusy* (1968) *via* an umbilical artery as indicated in Fig. 1.

The blood bathing the placenta together with the perfusate collected from this bath (indicated by *E* in Fig. 1) will be designated as »maternal perfusate«, whereas the perfusate collected from the foetus *via* the other umbilical artery (indicated by D) will be called »foetal perfusate« (F). It should be realized that whereas in this system the perfused material reaches the foetus from the placenta *via* the umbilical vein, there

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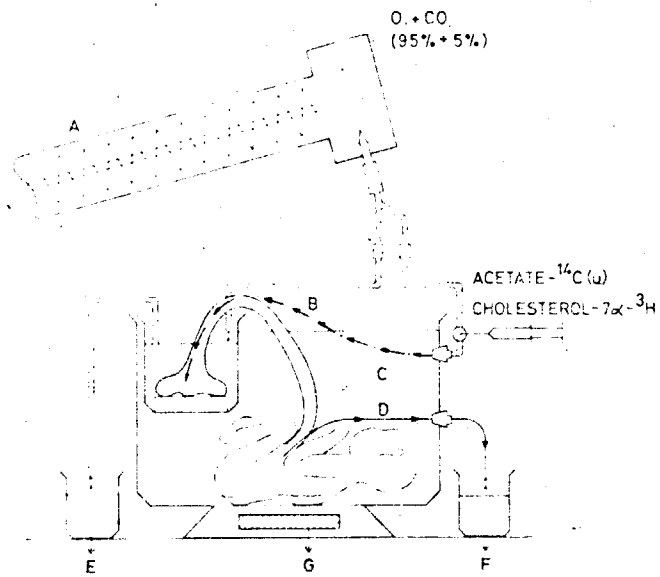

Fig. 1.

System for perfusion of the complete foeto-placental unit at midpregnancy according to *Lerner & Diczfalusy* (1968). A: oxygenator for the blood to be perfused. B: perfusion of the foeto-placental system *via* an umbilical artery. C: artificial amniotic fluid (saline-glucose). D: perfusate collected from the foetus *via* the other umbilical artery. E: perfusate collected from the blood bathing the placenta (»maternal perfusate«), F: container in which foetal perfusate is collected, and G: heater.

is no recirculation from the foetus to the placenta, since both umbilical arteries are cannulated. For the separate perfusion of midgestation placentas or foetuses the same experimental conditions were used.

Abbreviations and trivial names. – CCD: countercurrent distribution, cholesterol: 5-cholesten-3β-ol, cholesterol dibromide: 5α,6β-dibromo cholestane-3β-ol, DPM: disintegrations per minute, K: partition coefficient, S. A.: specific activity.

Material perfused. – Uniformly labelled sodium acetate-^{14}C, CFA 228, batch 8, and CFA 229 batch 12 (with a specific activity of 32.9 mCi/mmol* and 46.0 mCi/mmol, respectively) was used without further purification. Also cholesterol-7α-^{3}H (TRA 122 batch 7 and TRK 122 batch 6 with a S. A. of 500 mCi/mmol* was used without further purification. Its radiochemical purity was controlled by crystallisation from methanol and ethyl acetate, it was more than 98 % pure.

Administration of labelled material. – For the perfusion of the foeto-placental unit 5.0 mCi of sodium acetate-^{14}C were dissolved in 10 ml of saline and 10 ml of blood; 5.0 mCi of cholesterol-7α-^{3}H was dissolved in 0.5 ml of propylene glycol and 0.5 ml of abs. ethanol and 20 ml of 50 % diluted blood with saline. The two solutions were mixed and delivered by a perfusion pump at a constant rate (0.44 ml/min) into a polyethylene tube, through which the oxygenated blood was running p[illegible]ntering the umbilical artery. For the perfusion of the isolated foetus or placen[illegible] half of the above mentioned dose was used in half of the volume.

Extraction and separation of cholesterol. – The orginal purpose of the series of investigations to be reported in this and subsequent communications was the study of the steroids formed from acetate and cholesterol and the extraction method has been devised accordingly. Thus it does not represent optimal conditions for the extraction of cholesterol.

Upon completion of the perfusions the tissues were disintegrated in a Waring blender and extracted by 5 volumes of an ethanol-acetone (3:2) mixture. This was followed by extraction with one volume of acetone and 3 × 1 volumes of 80 % (v/v) ethanol. The following tissues were analysed separately: placentas, »maternal perfusates«, foetal livers, adrenals, residual foetal tissues and foetal perfusates.

The extracts were combined and evaporated almost to dryness at a temperature not exceeding 40°C and under reduced pressure. The residue was dissolved in 70 % (v/v) methanol and insoluble material precipitated according to the procedure described by *Mikhail et al.* (1963). The supernatant was evaporated to dryness as above and subjected to a 24-transfer countercurrent distribution between n-hexane and 90 % (v/v) methanol. The partition coefficient of cholesterol in this system is $K = 6.7$ whereas that of the least polar steroid studied (progesterone) is 0.12.

The contents of tubes 0–12 (»steroid fraction«) were pooled for the purposes of steroid analyses (to be reported in subsequent communications), whereas the contents of tubes 13–24 (containing cholesterol) were combined and evaporated to dryness. The residue was dissolved in a small volume of ethyl acetate and the material was subjected to thin-layer chromatography, in benzene:ethyl acetate (5:1) according to *Avigan et al.* (1963). In this way squalene and lanosterol were separated from cholesterol. The former two compounds were not studied further in the present investigation. Cholesterol was eluted with chloroform and in each case an aliquot of it was crystallised to constant specific activity, first as such and then as the dibromide. The cholesterol used for crystallisations was analytical grade from Merck AG-Darmstadt, Germany. Before use its was recrystallised twice (melting point: 148°C). The formation of cholesterol dibromide was carried out according to *Schwenk & Werthessen* (1952), as modified by *Kahara & McLaughlin* (1961).

Measurement of radioactivity. – This was carried out as described by *Mancuso et al.* (1965). All results are expressed in DPM.

RESULTS

Distribution of radioactive material. – Table 1 indicates the distribution of radioactive material in experiments No. 1 and 2: Table 2 indicates that of experiments No. 3 and 4 and finally Table 3 shows the data obtained in experiments No. 5 and 6.

It appears from the data of Tables 1–3 that irrespective of the type of experiment, significantly less ^{14}C than ^{3}H-label was recovered. The average recovery of ^{14}C in the six experiments was 35 %, with a range from 22 to 57 % whereas the mean recovery of ^{3}H was 82 %, with a range of 71 to 94 %. Following the precipitation of lipids in 70 % methanol at –17°C a major loss

* Purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England.

Table 1.

Distribution of radioactive material following the perfusion of two midgestation foetuses with 5 mCi of ^{14}C-labelled acetate and 5 mCi of ^{3}H-labelled cholesterol. Figures indicate percentage of the administered dose.

Tissues	Experiment No. 1												Experiment No. 2											
	Total recovery		Following 70 % (v/v) methanol precipitation		Following CCD								Total recovery		Following 70 % (v/v) methanol precipitation		Following CCD							
					»Sterol«		»Steroid«										»Sterol«		»Steroid«					
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Liver	0.55	2.17	0.54	1.18	0.29	1.95	0.25	0.13	0.33	0.57	0.22	0.22	0.003	0.06	0.22	0.16
Adrenal	0.54	0.37	0.20	0.33	0.04	0.19	0.16	0.14	0.36	0.29	0.36	0.20	0.13	0.08	0.23	0.12
Testis	0.067	0.009	0.006	0.009	0.003	0.008	0.003	0.001	0.003	0.001	0.002	0.001	<0.001	<0.001	<0.001	<0.001
Residual foetal tissues	3.20	4.60	2.06	1.11	0.04	0.34	2.01	0.77	5.55	8.70	5.54	1.33	0.003	0.04	5.54	1.29
Foetal perfusate	5.66	39.70	5.66	0.92	0.002	0.15	5.66	0.77	1.71	8.99	1.49	0.80	0.002	0.45	1.49	0.32
Total[a]	9.95	46.84	8.46	3.54	0.37	1.73	8.08	1.81	7.95	18.46	7.61	2.55	0.13	0.66	7.45	1.89
Placenta	13.52	29.37	10.66	6.65	0.21	2.00	10.45	4.65	11.96	32.18	8.07	5.44	1.00	0.63	7.07	4.81
Maternal perfusate[b]	1.82	2.89	1.24	0.43	0.006	0.10	1.24	0.33	2.32	0.58	2.31	0.41	0.001	<0.001	2.31	0.41
Total[a]	15.34	32.26	11.90	7.08	0.21	2.10	11.68	4.98	14.28	32.71	10.38	5.85	1.00	0.63	9.38	5.22
Grand total[a]	25.29	79.10	20.36	10.61	0.58	3.83	19.76	6.79	22.23	71.17	17.99	8.40	1.13	1.29	16.86	7.11

a) Figures less than 0.01 % are not included in totals.
b) Represents the blood bathing the placenta together with the perfusate collected from this bath.

Table 2.

Distribution of the radioactive material following the perfusion of two midgestation foetuses with 2.5 mCi of ^{14}C-labelled acetate and 2.5 mCi of ^{3}H-labelled cholesterol. Figures indicate percentage of the administered dose.

Exp.	Tissues	Total recovery		Following 70 % (v/v) methanol precipitation		Following CCD			
						»Sterol«		»Steroid«	
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
No. 3	Liver	5.98	10.99	3.82	7.41	0.68	5.89	3.14	1.52
	Adrenal	0.37	0.24	0.14	0.10	0.00	0.06	0.05	0.04
	Testis	0.01	0.004	0.01	0.04	0.005	0.002	0.005	0.002
	Residual foetal tissues	8.47	37.26	8.40	1.98	0.06	0.004	8.34	1.93
	Foetal perfusate	12.69	24.47	9.53	7.20	0.005	4.75	9.53	2.45
	Total[a]	26.62	73.06	21.90	16.69	0.83	10.70	21.06	5.94
No. 4	Liver	5.42	29.34	3.39	12.69	0.63	11.27	2.76	1.42
	Adrenal	1.06	0.55	0.82	0.46	0.26	0.22	0.56	0.24
	Testis	0.19	0.06	0.02	0.006	0.01	0.003	0.01	0.003
	Residual foetal tissues	17.12	27.00	8.93	4.60	0.02	0.39	8.91	4.21
	Foetal perfusate	19.24	32.47	19.24	2.71	0.001	0.19	19.24	2.52
	Total[a]	43.03	89.42	32.40	20.46	0.92	12.07	31.48	8.89

a) Figures less than 0.01 % are not included in totals.

occurred in both labels; however, whereas 67 % of the ^{14}C-labelled material present in the original ethanol-acetone extract was recovered after precipitation, the corresponding figure for the ^{3}H-label was only 17 %. In other words, whereas 24 % of the administered ^{14}C-labelled material (derived from acetate) was available for further processing following methanol precipitation, this was only associated with 14 % of the ^{3}H-labelled material administered, representing cholesterol and its metabolites.

The data also indicate that the relative distribution of the administered radioactive material among various tissues was significantly influenced by the type of experimental approach adopted. Thus, whereas following foetal perfusion (Experiment No. 3 and 4; Table 2) some 5 and 14 times more ^{14}C and 46 to 53 times more ^{3}H-labelled material was recovered from the liver than

Table 3.

Distribution of the radioactive material following the perfusion of two midgestation placentas with 2.5 mCi of ^{14}C-labelled acetate and 2.5 mCi of ^{3}H-labelled cholesterol. Figures indicate percentage of the administered dose.

Exp.	Tissues	Total recovery		Following 70 % (v/v) methanol precipitation		Following CCD			
						»Sterol«		»Steroid«	
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
No. 5	Placenta	21.41	66.69	11.95	12.33	0.26	5.95	11.69	6.38
	Maternal perfusate[b]	0.22	0.22	0.10	0.22	<0.001	<0.001	0.10	0.22
	Placental perfusate	35.98	26.68	35.98	1.94	0.27	<0.001	35.71	1.66
	Total[a]	57.21	93.59	48.03	14.49	0.53	5.95	47.50	8.26
No. 6	Placenta	14.90	26.19	12.76	7.81	0.14	1.52	12.62	6.29
	Maternal perfusate[b]	0.20	0.29	0.16	0.29	<0.001	0.002	0.16	0.29
	Placental perfusate	23.39	56.20	23.39	7.47	0.001	2.02	23.39	4.85
	Total[a]	38.49	84.68	36.31	15.57	0.14	4.14	36.17	11.43

[a] Figures less than 0.01 % are not included in totals.
[b] Represents the blood bathing the placenta together with the perfusate collected from this bath.

from the adrenals, in experiments No. 1 and 2 (Table 1) involving the perfusion of the complete foeto-placental unit, an equal amount of ^{14}C-labelled material was recovered from the livers and adrenals and the ^{3}H-labelled material recovered from the liver exceeded that found in the adrenals only some 3 to times.

A detailed analysis of the so-called »steroid-fraction« (which contains non-steroidal material as well) is reported in the following communications *Telegdy et al.* 1970*a,b*).

Isolation of cholesterol from various tissues. – Cholesterol was isolated in a radiochemically homogeneous crystalline form from all tissues studied in all experiments as shown in Tables 1–3. The crystallisation data of the cholesterol isolated from the 4 placentas (from experiments 1, 2, 5 and 6) are presented in Table 4.

Table 4.

Crystallisation of cholesterol isolated from the placentas in four experiments. Figures indicate DPM/mg cholesterol.

Crystals	Exp. No. 1		Exp. No. 2		Exp. No. 6		Exp. No. 7	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Starting material	822	532 700	870	176 000	240	412 300	711	758 400
Solvents[a]								
1.	115	526 400	78	176 200	56	409 400	0	733 000
2.	51	501 300	23	169 600	50	394 600	0	707 900
3.	56	542 500	0	176 900	36	462 900	0	753 100
4.	39	541 200	0	172 500	15	434 200	0	741 100
5.	21	539 400						
6.[b]	0	509 700			0	388 900		
7.[b]	0	502 500			0	394 900		
8.[b]	0	511 000			0	381 400		
Mother liquors								
1.	1940	521 300	5870	217 600	1870	45 400	4840	718 000
2.	145	587 500	312	179 300	30	432 600	5	737 600
3.	597	511 400	139	172 800	537	432 200	630	711 200
4.	143	554 900	12	177 400	119	411 700	0	724 800
5.	51	519 700						
6.[b]	–[c]	–[c]			–[c]	–[c]		
7.[b]	–[c]	–[c]			–[c]	–[c]		
8.[b]	–[c]	–[c]			–[c]	–[c]		

[a] 1. Methanol. 2. Ethanol. 3. Methanol-ethyl acetate. 4. Acetone-water. 5. Methanol. 6. Methanol-ethyl acetate. 7. Ethanol. 8. Methanol-acetone.
[b] Crystallised as cholesterol dibromide.
[c] Insufficient material.

It appears from the data of Table 4 that radiochemical homogeneity of the ^{3}H-label was achieved in each case and that all ^{14}C-label was completely dissociated from the ^{3}H-label prior or subsequent to bromination.

The crystallisation data of the cholesterol isolated from the placental perfusates (Experiments 5 and 6) are shown in Table 5.

The data of Table 5 indicate the absence of any ^{14}C-label from the cholesterol isolated. Cholesterol was also isolated in a radiochemically homogeneous form from the »maternal perfusates« of experiments No. 1, 2, 5 and 6. The crystalline cholesterol was devoid of any ^{14}C-labelled material in 3 out of 4 cases. In the fourth case (Exp. No. 6) a few counts of ^{14}C (between 15 and

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Table 5.

Crystallisation of the cholesterol isolated from the perfusates of two placentas. Figures indicate DPM/mg cholesterol.

Crystals	Experiment No. 5		Experiment No. 6	
	^{14}C	^{3}H	^{14}C	^{3}H
Starting material	53	160 300	0	589 900
Solvents[a)]				
1.	0	142 900	0	599 500
2.	0	135 500	0	489 000
3.	0	141 500	0	586 400
4.	0	134 000	0	591 000
Mother liquors				
1.	417	245 400	272	630 500
2.	0	156 400	0	577 300
3.	0	147 900	0	569 300
4.	0	140 900	0	588 500

a) 1. Methanol. 2. Ethanol. 3. Methanol-ethyl acetate. 4. Acetone-water.

23 DPM/mg) were present in the crystals following bromination: however, these values were still dropping and radiochemical homogeneity could not be established with regard to the ^{14}C-labelled material.

Typical examples of the crystallisation of cholesterol isolated from various foetal tissues are presented in Table 6.

It appears from the data that the cholesterol isolated from the liver (Exp. No. 1), adrenal (Exp. No. 2), testis (Exp. No. 4) and residual foetal tissues (Exp. No. 4) invariably contained ^{14}C-labelled material in a radiochemically homogeneous form. Cholesterol isolated from these four types of tissues in all experiments contained ^{14}C-label, except the carcass of Exp. No. 3. The quantitative significance of the ^{14}C-labelled cholesterol isolated from these tissues can be assessed on the basis of the data shown in Table 7.

Cholesterol was also isolated in a crystalline form from all »foetal perfusates« (Experiments 1–4). Radiochemical homogeneity was established with regard to the ^{3}H-label in all cases. However, as far as the ^{14}C-label is concerned, only the cholesterol isolated from the perfusate of experiment No. 4 was radiochemically homogeneous.

Amount of labelled cholesterol isolated from various sources. – The amounts of ^{14}C- and ^{3}H-labelled cholesterol isolated from the various foetal tissues and the $^{14}C/^{3}H$-ratios are presented in Table 7.

Table 6.

Crystallisation of the cholesterol isolated from various foetal tissues. Figures indicate DPM/mg cholesterol.

Crystals	Liver (Exp. No. 1)		Adrenal (Exp. No. 2)		Testis (Exp. No. 4)		Residual foetal tissues (Exp. No. 4)	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Starting material	1890	28 700	4770	14 400	2800	1480	968	71 300
Solvents[a)]								
1.	1500	24 200	3170	13 600	2270	1290	428	71 300
2.	1580	24 000	3380	13 300	2260	1250	393	69 500
3.	1380	25 300	2670	13 800	2370	1360	372	71 700
4.	1210	24 890	2840	13 900	2110	1280	296	72 100
5.	1070	23 900	2420	13 400	1970	1250		
6.[b)]	979	23 800	2120	18 200	1890	1210	137	72 200
7.[b)]	938	22 800	2090	13 100	1890	1200	103	60 000
8.[b)]	986	23 900	2180	18 200	1920	1230	111	72 000
9.[b)]	960	23 600					110	66 000
Mother liquors								
1.	5250	25 100	10 100	15 500	5370	2010	3070	67 000
2.	1290	24 900	2830	13 700	2050	1260	566	71 800
3.	4310	23 000	9780	14 800	2500	1380	819	71 800
4.	1870	24 400	2630	13 400	3850	1500	868	72 200
5.	1550	23 500	4250	13 900	2840	1460		
6.[b)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]
7.[b)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]
8.[b)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]
9.[b)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]	–[c)]

a) 1. Methanol, 2. Ethanol, 3. Methanol-ethyl acetate, 4. Acetone-water, 5. Methanol, 6. Methanol-ethyl acetate, 7. Ethanol, 8. Methanol-acetone, 9. Methanol-ethyl acetate.
b) Crystallised as cholesterol dibromide.
c) Insufficient material.

These figures have been calculated on the basis of the S. A. of the last two crystals of the cholesterol dibromide and were not corrected for procedural losses. It appears from the data that there is a considerable variation in the amount of cholesterol synthesized from acetate in the various experiments. The $^{14}C/^{3}H$-ratio is highest in the cholesterol isolated from the adrenals and testicles and lowest in the cholesterol of the residual foetal tissues. Furthermore, more than 98 % of the total ^{14}C-labelled cholesterol recovered was

Table 7.

^{14}C- and ^{3}H-labelled cholesterol isolated from various foetal tissues. The figures indicate DPM and were not corrected for procedural losses. The ^{14}C to ^{3}H ratio of the isolated cholesterol is also indicated.

Tissues		Exp. No. 1	Exp. No. 2	Exp. No. 3	Exp. No. 4	Total
Liver	^{14}C	3 297 000	6619	8 006 000	14 238 000	25 547 619
	^{3}H	76 700 000	3 610 000	178 478 000	490 160 000	748 948 000
	$^{14}C/^{3}H$	0.043	0.002	0.045	0.029	0.034
Adrenal	^{14}C	30 610	544 500	656 400	5 443 000	6 674 510
	^{3}H	11 994 000	3 255 000	3 140 000	11 546 000	29 935 000
	$^{14}C/^{3}H$	0.003	0.167	0.209	0.471	0.223
Testis	^{14}C	51 870	564	1763	193 100	247 296
	^{3}H	768 500	75 360	19 680	123 300	968 840
	$^{14}C/^{3}H$	0.067	0.007	0.089	1566	0.250
Residual foetal tissues	^{14}C	158 300	666	0	22 060	181 026
	^{3}H	27 540 000	1 817 000	1 860 000	17 314 000	48 531 000
	$^{14}C/^{3}H$	0.006	0.004	0.000	0.001	0.004
Total	^{14}C	3 537 780	552 349	8 664 162	19 896 160[a]	32 650 451
	^{3}H	117 002 500	8 757 360	183 497 680	519 143 300	828 400 840
	$^{14}C/^{3}H$	0.039	0.063	0.047	0.038	0.039

[a] In addition, in this experiment 5029 DPM ^{14}C-labelled and 591 000 DPM ^{3}H-labelled cholesterol was also isolated from the perfusate.

isolated from the liver and adrenals. Finally, the over-all $^{14}C/^{3}H$-ratio of the cholesterol isolated from all foetal tissues was approximately 0.04.

DISCUSSION

It has been reported that minced term placentas convert labelled acetate to squalene, lanosterol (*Villee & Tsai* 1969) and to small amounts of cholesterol (*van Leusden & Villee* 1965; *Zelewski & Villee* 1966). A minute conversion of acetate to cholesterol was also demonstrated following the perfusion of term placental cotyledons (*Levitz et al.* 1962). On the other hand, the data reported by *Hellig et al.* (1968) indicate, that if the term placenta *in situ* synthesizes cholesterol at all, its quantity must be minimal indeed.

The data reported in this investigation indicate, that the midgestation human placenta perfused with or without the foetus is not capable of converting any acetate to cholesterol. In a forthcoming communication further data will be presented indicating that all steroid metabolites isolated from the placentas in experiments No. 1, 2, 5 and 6 were devoid of any ^{14}C-label (*Telegdy et al.* 1976*a*). In view of these data it is concluded, that the midgestation human placenta is not capable of synthesizing cholesterol from acetate. On the other hand, large quantities of ^{14}C-labelled squalene- and lanosterol-like material were found in the extracts of all placentas studied. Thus it appears possible, that the midgestation placenta is capable of synthesizing considerable quantities of lanosterol, but is not able to convert this lanosterol to cholesterol. Further studies will be needed to find out whether or not the squalene and lanosterol formed by the placenta are secreted to the foetus, and if so, whether the foetal organism is utilizing these precursors for cholesterol synthesis.

It has been reported that acetate is incorporated into cholesterol by foetal adrenal slices (*Bloch & Bonirschke* 1962). Labelled cholesterol was also isolated from the foetal adrenal and liver following the perfusion of two foetuses with labelled acetate (*Solomon et al.* 1967) and the incorporation of labelled acetate into foetal adrenal and liver tissue was also described following the administration of large amounts of labelled acetate to mothers prior to the interruption of gestation (*Davis et al.* 1956; *Plotz et al.* 1968).

The results presented in this paper confirm and extend these earlier observations and indicate that the foetal liver, adrenals and testicles are capable of converting labelled acetate to cholesterol. The extent of conversion of acetate to cholesterol by the testicles of perfused foetuses showed considerable individual variations. However, in one case (Exp. No. 4) it was very extensive and the $^{14}C/^{3}H$-ratio of the isolated cholesterol was the highest among all tissues studied. ^{14}C-Labelled cholesterol was also isolated from the residual foetal tissues in 3 of 4 experiments, although in much smaller amounts than those present in the liver and adrenals. Whether this cholesterol was synthesized by foetal tissues such as the skin or brain (*e. g. Miettinen & Luukkainen* 1968), or whether labelled cholesterol was secreted by the liver and/or adrenal to other foetal tissues, remains to be determined. The isolation of ^{14}C-labelled cholesterol from one of the four perfusates suggests secretion of some of the synthesized cholesterol.

The quantitatively most important sources of cholesterol synthesis were the liver and adrenals. More than 98 % of the total ^{14}C-labelled cholesterol present in the foetuses was isolated from these two organs. In view of the major losses of cholesterol during the 70 % methanol precipitation stage (cf. Tables 1–3) the conclusion seems to be justified that in the organism of the midgestation human foetus the *de novo* cholesterol synthesis from acetate is a quantitatively important pathway. Evidence will be presented in a forthcoming communica-

tion to indicate that this cholesterol is extensively utilized by the foetus for the synthesis of various steroids (*Telegdy et al.* 1970*b*).

From the over-all $^{14}C/^{3}H$-ratios of the cholesterol isolated in the present study (Table 7) it would be tempting to conclude that some 4 % of the cholesterol isolated was newly synthesized from acetate. However, such an assumption is based on a major uncertainly concerning the number of ^{14}C-atoms which were incorporated into each cholesterol molecule synthesized.

Finally, it is of interest to note that the uptake of ^{14}C-labelled material in relation to that of ^{3}H-labelled material was highest in the adrenals, suggesting an extensive metabolism of acetate in this organ. The significance of this in relation to the steroid synthesis of the foetal adrenals will be discussed in a forthcoming communication (*Telegdy et al.* 1970*b*).

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Testicular Sterols

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I. Incorporation of Mevalonate and Acetate into Sterols by Testicular Tissue from Rats[1]

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Conditions have been established for the incorporation of labeled acetate and mevalonate into sterols of rat testicular tissue. Maximal rates of incorporation into the nonsaponifiable lipids were obtained with phosphate buffer (0.05 *M*, pH 7.4), sodium chloride (0.05 *M*), glucose (0.033 *M*), potassium (0.021 *M*), magnesium (0.006 *M*), suspended testicular tissue (approximately 50–75 mg. of protein), and acetate (0.0028 *M*) or D,L-mevalonate (0.00050 *M*). Unlabeled mevalonate (2.7×10^{-4}–2.7×10^{-3} *M*) depressed the incorporation of labeled acetate (3.2×10^{-3} *M*). Labeled lanosterol, squalene, cholesterol, and acyclic alcohols (farnesol and nerolidol) were purified with unlabeled carriers. In addition, small amounts of activity were associated with minor fractions of sterols (possibly 24,25-dihydrolanosterol and Δ^7-cholestenol). After incubation with acetate-1-C^{14} *in vitro*, relative amounts of activity in the various fractions of the nonsaponifiable lipids were: squalene, 34%; lanosterol and companion C_{30}-, C_{29}-, and C_{28} sterols, 19%; cholesterol and companion C_{27}-sterols, 16%; and unfractionated steroids, 26%. After incubation with mevalonate-2-C^{14}, the relative activities were 32, 21, 12, and 8%, respectively, of the total label that was isolated in the nonsaponifiable fraction. Labeled acetate was incorporated into all fractions of testicular sterols *in vivo*. Labeled acetate and mevalonate were not incorporated into sterols by cell-free homogenates of testicular tissue in fortified media; similar homogenates metabolized labeled lanosterol.

Labeled acetate is incorporated into sterols by testicular tissue *in vitro* (1–3). Conversion of cholesterol[2] into testosterone and other C_{19}-steroids by homogenates of testicular tissue (4) or by incubation *in vivo* (5) has been demonstrated. Two additional lines of evidence suggest that the synthesis of sterols in testicular tissue may have particular metabolic significance. First, anomalous specific radioactivities of crude digitonin precipitable sterols from incubation with "labeled" substrates have led to the suggestion that cholesterol may not be an obligatory precursor of testosterone (2, 6, 7). Second, Morris and Chaikoff (8) concluded that most testicular cholesterol is formed *in situ* and not transferred from blood plasma. Accordingly, the synthesis of testicular sterols, the identification of the sterols, and the role of the sterols as precursors of steroid hormones are under investigation. This initial report describes conditions for the incorporation of labeled acetate and mevalonate into testicular sterols and the identification of the principal labeled products.

[1] Supported in part by funds from the State University of New York and by Grant G-19556 from the National Science Foundation.

[2] Common names of sterols are referred to in this report: cholesterol is cholest-5-en-3β-ol; lanosterol is 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; Δ^7-cholestenol is 5α-cholest-7-en-3β-ol.

MATERIALS AND METHODS

Incubation Procedure

Testicular tissue from adult rats was dissected, the surrounding membrane was removed, and the tissue was cut with scissors. The tissue was suspended uniformly in phosphate (0.05 *M*) and sodium chloride (0.05 *M*) buffer (9) or Krebs bicarbonate buffer (10). Each buffer was pH 7.4 and contained Mg^{++} (0.006 *M*) and K^{+} (0.024 *M*). Samples of the suspension of testicular tissue that contained approximately 50-75 mg. of protein were transferred to Erlenmeyer flasks. Sodium salts of the substrates were added, and the final volume was adjusted to 2.5-4.0 ml. The flasks were flushed with oxygen, stoppered, and incubated for 3 hours at 37°C. with shaking. At the end of the incubation period, 2.5-4.0 ml. of alcohol and 1 g. of KOH were added to each flask. The mixture was heated under reflux for 4 hours. An equal volume of water was added to the cooled samples, and the nonsaponifiable lipid was extracted twice with diethyl ether (60 ml. total) and once with 20 ml. of chloroform. The extract was washed with water and dried over sodium sulfate. The residues from the extract were counted with a liquid scintillation spectrometer (counting efficiency of 70% for C^{14}).

After homogenization in water, protein was determined by the method of Lowry *et al.* (11). In some incubations, proportionally larger amounts of tissue and labeled substrates were used. These variations are indicated in the text, tables, and figures.

Fractionation of the Nonsaponifiable Lipids

The residue from the extract of nonsaponifiable lipids was dissolved in 40 ml. of pentane, and the solution was extracted twice with 40 ml. of 90% of methanol in water (v/v). The ratio of authentic Δ^5-cholestenol,[3] lanosterol,[3] and testosterone[3] in the pentane to that in the aqueous methanol was 4:1, 7:1, and 1:37, respectively. Accordingly, the methanol fraction contained primarily a mixture of polar steroids, and the pentane fraction contained a mixture of sterols, squalene, and other lipids of low polarities. Steroids were fractionated chromatographically with columns of alumina (2) or with silica gel on thin layer plates (12). The residue from the pentane was fractionated chromatographically with columns of deactivated alumina (13, 14). Three major fractions were collected: squalene; lanosterol and companion C_{30}-, C_{29}-, and C_{28} sterols; and cholesterol and companion C_{27}-sterols. Specific and more detailed fractionation procedures are given in the text. Δ^4-3-Ketosteroids were determined spectrophotometrically in alcohol solution (λ_{max} 238 mμ), and sterols were determined with a modified Liebermann-Burchard procedure (14).

Partial Separation of Testicular Tissue

Whole testicular tissue was teased and suspended in phosphate-sodium chloride buffer. The suspension was filtered on four layers of cheese cloth. The retained cells from the filtration were suspended in phosphate or bicarbonate buffers. Appropriate samples of the whole tissue or the separated tissue were distributed into incubation flasks to yield final volumes of 3.1, 3.1, and 2.6 ml of suspension in flasks that contained whole tissue, retained cells, and filtered cells, respectively. The samples were assayed for protein and activity of alkaline phosphatase (15) after homogenization. Approximately 63% of the total alkaline phosphatase activity was in the filtrate. Histochemically, much of the testicular alkaline phosphatase is localized in the interstitial cells (16).

RESULTS

Effect of Buffer

Suspensions of whole testicular tissue, retained tissue, or filtered cells (56, 115, and 25 mg. of protein, respectively) were incubated with phosphate-NaCl buffer or bicarbonate buffers which contained 0.6 μc of acetate-1-C^{14} in 720 mμmoles.[4] Between 1.31 and 1.64 times the amount of radio-

[3] Cholesterol was purified as the dibromide (17) (m.p. 147°-148°; $[\alpha]_D$ -38.5° $CHCl_3$, *c*, 0.6); lanosterol was purified as the 24,25-dibromo derivative (21) (m.p. 137°-139°; $[\alpha]_D$ +60.1°, $CHCl_3$, *c*, 0.3); 24,25-dihydrolanosterol was prepared by hydrogenation of lanosterol with a suspension of Raney nickel in ethanol; testosterone (Lot T 11B-94, Sigma Chemical Co., St. Louis, Mo.) was recrystallized from ethanol; Δ^5-cholestenol was prepared by catalytic reduction of 7-dehydrocholesterol in dioxane solution with Raney nickel (m.p. 122°-123°; $[\alpha]_D$ +2.2°, $CHCl_3$, *c*, 0.6); commercial farnesol (Aldrich Chemical Co., Milwaukee, Wisconsin) was used without further purification. Distillation at a reduced pressure yielded only farnesol; farnesal was obtained from commercial nerolidol (K and K Laboratories, Jamaica, New York) by distillation at a reduced pressure (89°-91°, 0.4 mm Hg); geraniol and squalene were purchased from Distillation Products, Inc., Rochester, New York (each compound was distilled at a reduced pressure).

[4] New England Nuclear Corp., Cambridge, Massachusetts.

activity was incorporated into crude nonsaponifiable lipids from phosphate buffer than from bicarbonate buffer with each of the three preparations of tissue. Phosphate-NaCl buffer (with Mg^{++} and K^{+}) was used in the subsequent experiments. The relative radioactivities of the nonsaponifiable lipids were 13.9, 7.7, and 12.4 cpm. per milligram protein for whole, retained, and filtered cells, respectively.

Effect of Substrate Concentration

Acetate-1-C^{14} (1 μc in 0.2–10 μmoles per flask) was added to suspensions of whole testicular tissue in phosphate buffer. The effect of concentration on the incorporation of labeled acetate was essentially linear (Fig. 1). Maximal counting rates of the nonsaponifiable lipids were obtained from flasks that contained solutions of intermediate concentrations of acetate (approximately 1–4 μmole per flask).

Mevalonic acid (0.1 μc in 0.075–1.875 μmoles of D,L-mevalonate)[1] was incubated with similar suspensions of whole testicular tissue. The response to increased concentrations of substrate was linear over the range of concentrations that was investigated (Fig. 1). Maximal counting rates were obtained with dilute solutions (approximately 0.15 μmoles per flask).

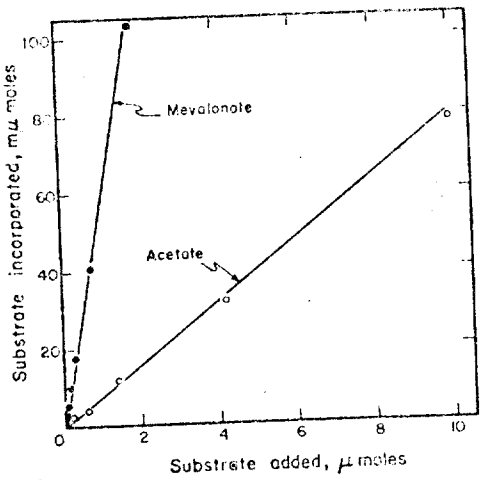

FIG. 1. Effect of acetate and mevalonate concentration on the formation of nonsaponifiable lipids in testicular tissue. Each flask contained whole testicular tissue (approximately 50 mg. of protein), phosphate buffer, and various amounts of labeled acetic acid (1.0 μc) or labeled mevalonic acid (0.1 μc) in a final volume of 3.5–3.8 ml. The total nonsaponifiable fraction was assayed for radioactivity. Each value represents the average of 6 samples from 3 separate experiments.

TABLE I

EFFECT OF GLUCOSE CONCENTRATION ON THE INCORPORATION OF ACETATE

Each flask contained approximately 75 mg. of protein from whole testicular tissue and acetate-1-C^{14} (0.6 μc in 720 mμmoles). The final volume was 3.8 ml. The samples were incubated for 3 hours at 37°C. The entire nonsaponifiable fraction was extracted and counted.

Glucose added (μmoles)	No. samples	Average incorporation (cpm./mg. protein[a])
0	3	26
5	3	58
25	4	70
50	3	137
125	4	144
250	3	152

[a] Protein was determined following homogenization of the contents of each flask after the incubation.

Suspensions of whole testicular tissue were incubated with glucose (0–250 μmoles per flask) and acetate-1-C^{14} (0.6 μc in 720 mμmoles). The incorporation of acetate was increased by 5 μmoles or more of glucose per flask (Table I). The incorporation was not altered by a fivefold increase in the concentration of glucose that produced maximal rates of incorporation (50 μmoles). For incubation, glucose (50–125 μmoles per flask) was added to all preparations of tissue.

Suspensions of whole tissue were incubated with labeled acetate, glucose, and various amounts of unlabeled mevalonic acid (0.1–100 mμmoles per flask). Incorporation of acetate into the nonsaponifiable lipids was inhibited approximately 20% by 1.0 mμmole of unlabeled mevalonate (Table II). The addition of more unlabeled mevalonate resulted in further inhibition.

Gross Fractionation of Labeled Sterols

Testicular tissue was suspended and filtered through cheese cloth. The retained

TABLE II

EFFECT OF UNLABELED MEVALONIC ACID ON THE INCORPORATION OF LABELED ACETATE

Each flask contained approximately 100 mg. of protein from whole tissue, 125 μmoles of glucose, and acetate-1-C^{14} (0.6 μc/120 mμmole). The crude nonsaponifiable lipids were extracted and counted (final volume, 3.7 ml.). Each value represents the means of 6 samples (and standard errors), except 1 sample (10 mμmoles) that was lost inadvertently.

Mevalonate added (mμmoles/flask)	Radioactivity isolated (cpm/mg. protein)	% Depression
0	221 ± 12	0
0.1	210 ± 12	5
1.0	181 ± 6	18[a]
10	174 ± 12	21[a]
100	166 ± 8	24[b]

[a] Statistically significant.
[b] Highly statistically significant.

cells were incubated with acetate-1-C^{14} as described previously. Sterols were obtained by chromatography on Merck alumina (13). The sterol fraction (approximately 8 mg.) was transferred to a column of silicic acid (1 × 15 cm.). The chromatogram was developed with an increasing concentration of benzene in hexane as described in the legend to Fig. 2. Fractions were collected and assayed for radioactivity and cholesterol. Most of the radioactivity was collected in fractions that did not contain cholesterol (Fig. 2). From this incubation, only a small amount of radioactivity was isolated with the endogenous cholesterol. Traces of yellow color formed in tubes 37–46 when the Liebermann-Burchard reagent was added. The yellow chromophore and the retention of lanosterol on this column of silicic acid corresponded (14). Lanosterol and companions (tubes 35–55) and cholesterol and companions (tubes 59–75) were separated by chromatography on deactivated alumina (14), or by chromatography on plates of silica gel (12) for the following procedures.

PURIFICATION OF LABELED CHOLESTEROL AND COMPANIONS

Whole testicular tissue (8 g.) was incubated with labeled acetate (8 μc) for 3 hours. The crude sterol fraction was separated into cholesterol and companions by chromatography on deactivated alumina. A total of 59.6 mg. of cholesterol was isolated in the crude cholesterol and companions. The specific activity was 670 cpm per milligram of cholesterol. Authentic cholesterol[3] was added (415 mg.), and the mixture was purified as outlined in Table III. The original mixture was treated with bromine (17). The product was recrystallized from acetone. The specific activity of the resulting dibromide was 29% of the original. The specific activity remained essentially constant during additional recrystallizations and debalogenation. The recrystallization was repeated with a total of 169 mg. of labeled cholesterol and companions (4430 cpm). The specific activity decreased from 26.2 to 9.4 cpm per milligram or 36% of the original specific activity. Slightly higher retention of radioactivity (43%) was obtained when labeled cholesterol and companions were purified from an incubation of acetate and testicular tissue of rats that

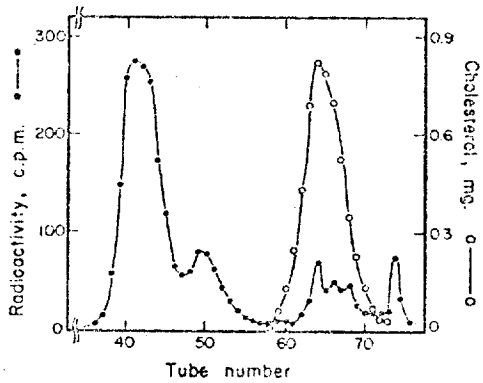

FIG. 2. Chromatographic resolution of labeled sterols. The sterol fraction from chromatography on Merck alumina was transferred to a column of silicic acid (1 × 15 cm., Mallinckrodt). The column was developed with an increasing gradient of 15–85% of benzene in hexane by replacing the effluent from the mixing flask (250 ml.) with 85% of benzene in hexane. Fractions were collected at intervals of 45 minutes (approximately 7.5 ml.). The residue in each fraction following evaporation *in vacuo* was assayed for sterol and radioactivity. Traces of sterols other than cholesterol were observed with the Liebermann-Burchard reagent; however, the concentrations were not adequate for accurate analysis.

TABLE III

PURIFICATION OF LABELED CHOLESTEROL WITH CARRIER

The labeled cholesterol and companion C_{27}-sterol fraction was isolated as described under *Materials and Methods*.

	Weight (mg.)	Specific activity (cpm/mg.)	% Original Specific activity
1. Isolated	59.6	670	–
2. Plus cholesterol	475	84.1	100
3. Dibromide	–	24.6[a]	29.2
4. Recryst. acetone	–	24.0[a]	28.6
5. Debrominated	248	24.0	28.6
6. Recryst. methanol	–	27.0	32.1

[a] The specific activity was corrected for changes in the molecular weight.

were injected three times weekly with 200 IU of human chorionic gonadotropin (Ayerst Laboratories, New York, New York).

Samples of cholesterol and companions with high specific activities were obtained from plates of silica gel (12). The crude fractions from 2 separate incubations (1.5 and 2.3 mg. cholesterol, respectively) were mixed with 1.1 mg. of authentic Δ^7-cholestenol.[3] The sterols were esterified with *p*-phenylazobenzoyl chloride, and the esters were chromatographed on columns of silicic acid (18). The silicic acid and absorbed steryl esters were extruded, and the Δ^7-zone (primarily esterified Δ^7-cholestenol) and the Δ^5-zone (primarily esterified cholesterol) were assayed for radioactivity and steryl ester (14). The Δ^5-zone contained 36 and 33% of the radioactivity that was recovered in the 2 zones. The experiment was repeated with a sample of cholesterol and companions from chromatography on deactivated alumina rather than from plates of silica gel; a lower percentage of radioactivity was isolated with cholesterol (24%).

PURIFICATION OF LABELED LANOSTEROL

Eight g. of whole testicular tissue was incubated with 8 μc of acetate-2-C^{14}. The nonsaponifiable lipids (83,500 cpm) were chromatographed on alumina, and three fractions were collected: hydrocarbons, 32,500 cpm; lanosterol and companions 35,500 cpm; and cholesterol and companions, 8400 cpm.

A sample of the labeled lanosterol and companions (7100 cpm) was mixed with 0.75 mg. of authentic lanosterol[3] and 2.0 mg. of 24,25-dihydrolanosterol.[3] The mixture was transferred to a column of deactivated alumina (1 × 15 cm.). A solution of 7.5% of benzene in hexane eluted the sterols. Samples of the effluent were assayed for sterol and radioactivity. Fractions of 24,25-dihydrolanosterol contained approximately 1770 cpm. Approximately 3690 cpm were eluted with the combined trailing edge of 24,25-dihydrolanosterol and lanosterol. Unlabeled lanosterol (5 mg.) was added to the chromatographic fractions that contained resolved lanosterol (2060 cpm). The mixture was acetylated and the product was chromatographed on silica gel (19). The specific activity of the eluted product was constant (calculated: 371 cpm per milligram maximally as the acetate; found: 343 cpm per milligram).

Unlabeled lanosterol (1 mg.) was added to crude lanosterol and companions (3550 cpm). The mixture was chromatographed on deactivated alumina (5 g.), and 3060 cpm were recovered. More carrier was added (approximately 34 mg.), and the mixture was recrystallized twice from acetone and methanol (Table IV). The specific activity of the crystalline product was 51–52% of the original sample. The specific activity of the crystals (calculated as unesterified sterol) remained unchanged following acetylation. The product was treated with osmium tetroxide (20), and the 24,25-dihydroxylanost-8-en-3β-ol acetate was isolated. The specific activity (calculated as lanosterol) was lowered slightly by purification. The product (approximately 450 cpm) was cleaved with lead tetraacetate (21), and acetone was collected by distillation (12.5 cpm) after the addition of unlabeled acetone. The products following distillation were extracted and chromatographed on deactivated alumina; the aldehyde (120 cpm) was eluted with 10% of benzene in hexane; unchanged dihydroxysterol (218 cpm) was

TABLE IV

RECRYSTALLIZATION OF LANOSTEROL AND COMPANIONS WITH UNLABELED LANOSTEROL

The labeled lanosterol and companions was isolated from testicular tissue following incubation with acetate-2-C^{14}.

Treatment	Specific activity[a] (cpm/mg.)	Original specific activity
1. Original	88.2	100
2. Recrystallized from acetone	44.6	51
and methanol	45.6	52
	47.0	53
3. Acetylated	45.5	52
Recrystallized	40.5	46
4. Treatment with Osmium tetroxide	41.3	47

[a] The specific activity was corrected for changes in the molecular weight.

recovered with 5% of ethanol in acetone. The ratio of activity in the aldehyde and acetone (120/12.5 = 9.6) agreed with the theoretical ratio of 8 within previously reported limits of experimental error and recovery of products (21).

Labeled lanosterol and companions from acetate-2-C^{14} (carrier-free, 3550 cpm) was dissolved in acetone, and 25 mg. of Tween 80[5] in acetone solution was added. The solution was heated to boiling and 5 ml. of warm 0.1 *M* phosphate buffer (pH 7.4) was added. The acetone was removed with a stream of nitrogen. Two 1-ml. samples of the suspension were incubated for 30 minutes with an homogenate of rat liver (21). Two 1-ml. samples were incubated similarly after the homogenate was heated at 100°C. for 3 minutes. Labeled carbon dioxide was collected (21). The yield of carbon activity in carbon dioxide was 84 cpm.; the heated control liberated less than 2 cpm. The yield of radioactivity in the C_{27}-sterols was 346 and 6.9 cpm. in samples of normal and heated homogenates, respectively. The ratio of activities in cholesterol and carbon dioxide (346/84 = 4.1) further suggests that labeled lanosterol was present. A theoretical ratio of 5.0 is expected (21).

PURIFICATION OF ACYCLIC ALCOHOLS

A shoulder or more defined peak of radioactivity associated with lanosterol (Fig. 2, tubes 47–52) and cholesterol (Fig. 2, tubes 73–75) was obtained repeatedly in varying proportions. The labeled species did not precipitate with digitonin. The labeled product from the supernatant fraction of the lanosterol and companions (1650 cpm) that was treated with digitonin was oxidized with an excess of 2 *N* chromic acid in acetic acid solution at room temperature. The crude product was extracted from an alkaline solution, the residue was heated 1 hour at 100° with a solution of 1% of Girard T reagent in ethanol-acetic acid solution (9:1), and the Girard-positive fraction was obtained (800 cpm) following extraction of the unchanged material from an acidified medium and neutralization of the solution. An identical treatment of a sample that was not oxidized before reaction with Girards reagent yielded 46 cpm. Attempted regeneration of the carbonyl group by hydrolysis with HCl was not successful (12 cpm).

The labeled fraction (7200 cpm) from the supernatant solution following precipitation of sterols from nonsaponifiable lipids with digitonin was extracted and suspended in phosphate buffer (pH 8.5, 0.01 *M*) by evaporation of a solution of the lipid in acetone. The sample was diluted, and 2-½ portions were incubated for 1 hour at 37°C. in 10 ml. of phosphate buffer, (pH 8.1; 0.01 *M*) 16 μmoles of NAD,[5] and 10 units of liver alcohol dehydrogenase.[6] The sample was treated with Girards reagent. An average of 236 cpm was obtained in the Girard positive extract of the two flasks. The heated control yielded 57 cpm. Incubation of similar preparations with yeast alcohol dehydrogenase[6] and bacterial steroid dehydrogenase[6] yielded no significant increase in the amount of activity in the carbonyl fraction. Others have reported that the latter two enzymes do not oxidize farnesol (22).

The pentane fraction from 2 separate incubations was treated with digitonin. The supernatant fraction was mixed with carrier

[5] Nutritional Biochemicals Corp., Cleveland, Ohio.

[6] Worthington Biochemical Corp., Freehold, New Jersey.

farnesol.[3] The original specific activities were 28.4 and 172 cpm per milligram (Table V). The preparations were esterified with *p*-phenylazobenzoyl chloride, and the esters were chromatographed on columns of silicic acid and celite (3:1) with benzene in hexane (1:5.5). The chromatography yielded broad bands of colored esters which were obtained by extrusion of the support with the absorbed ester and extraction of the ester from the support with ethanol and ether. The ester was saponified from the second experiment, and the alcohol was esterified again with 3-nitrophthalic anhydride. After repeated crystallizations, white needles were obtained. The final product contained 3.84 cpm per milligram (calculated as farnesol) or 2.2% of the original specific activity.

Labeled nonsaponifiable lipid was partitioned between methanol and pentane. The lanosterol and companion fraction (38,000 cpm) was mixed with 5.54 g. of carrier farnesol and the mixture was oxidized to farnesal with chromic acid according to the method of Ruzicka (24). The semicarbazide of the oxidized product was prepared and recrystallized from 50% ethanol in water to a constant specific activity of the crystals and mother liquor (2.2 cpm per milligram). The percentage of original specific activity was 32. This value represented approximately 15% of the original steroid-free mixture. Both nerolidol and farnesol yield farnesal upon chromic acid oxidation (21), hence a portion of the label may be ascribed to the previously undetected nerolidol. Purification with authentic farnesal semicarbazide did not affect the specific activity.

TABLE V

PURIFICATION WITH CARRIER FARNESOL

In experiment I the pentane extract was treated with digitonin, and the supernatant fraction (12,800 cpm) was mixed with 450 mg. of carrier farnesol. In experiment II, 38,300 cpm was mixed with 223.2 mg. of carrier. Additional carrier (2.1188 g.) was added after the formation of the *p*-phenylazobenzoyl ester. The specific activities were corrected for the various dilutions and derivative formations. In the second part of experiment II, 38,900 cpm from lanosterol fraction and 5.54 g. of farnesol were combined.

Derivative	Specific activities			
	Experiment I		Experiment II	
	cpm/mg.	%	cpm/mg.	%
1. Original	28.4	100	172	100
p-Phenylazobenzoate	2.72	9.6	16.1	9.4
3-Nitrophthalate	—	—	3.84	2.2
2. Original	—	—	6.85	100
Semicarbazide	—	—	2.20	32

PURIFICATION OF LABELED SQUALENE

A solution of labeled hydrocarbon (5700 cpm) from the column chromatographic separation of products from incubation with acetate-2-C^{14} was mixed with 854 mg. of carrier squalene.[3] The mixture was chromatographed on 50 g. of Merck alumina. The product was eluted with a total of 500 ml. of hexane (796 mg., 6.56 cpm per milligram). A portion of the crude mixture (approximately 400 mg.) was treated in chloroform solution for 30 minutes with dry HCl. The hexahydrochloride was recrystalized to a constant and equal specific activity of crystals and mother liquor 5.58 cpm per milligram (calculated as squalene).

The remaining sample of squalene was stirred for 3 days at room temperature with an equimolar amount of osmium tetroxide in 3 ml. of dry pyridine. The white product (208 mg.) with a specific activity of 5.12 cpm per milligram (calculated as squalene from the fully hydroxylated substance ($C_{30}H_{62}O_{12}$)) crystallized from absolute ethanol-hexane (3:1). After 3 crystallizations from the same solvent, the mother liquors were combined and the residue was chromatographed on acid washed alumina. Unchanged squalene was recovered (27.2 mg.) with a specific activity of 5.53 cpm per milligram. The polar compounds were collected with 25% of ethanol in acetone. The specific activity of the mixture (6.78 cpm per milligram as fully hydroxylated) indicated the presence of some intermediate products of hydroxylation. The crystalline dodecahydroxy compound following crystal

lization (76 mg., 5.45 cpm per milligram) was treated with lead tetraacetate as described previously (21). Carrier acetone was added, and a total of 78 cpm was collected by distillation (92 cpm, theoretical).

Relative Activity in the Various Chromatographic Fractions

The relative distribution of radioactivity in the labeled products from 11 separate incubations with acetate-1-C^{14} are recorded in Table VI. No exogenous carriers were added. Cholesterol, lanosterol, and squalene essentially were labeled equally. The total activity in the unfractionated steroid fraction was higher. Little variation was observed in the samples of crude extract, mixed squalene and sterols, squalene, and steroids. However, the ratio of radioactivity between lanosterol and cholesterol within the fraction of unfractionated sterols varied from 0.32 to 9.0 when the samples were separated with columns.

Samples of nonsaponifiable lipids from four similar experiments were separated by thin layer chromatography immediately following extraction. The relative distribution of activity was similar for steroids, lanosterol, and cholesterol. The recovery of the small amount of labile squalene was substantially greater by this more rapid technique. Separation on thin layer plates resulted in a decreased variation and improved recovery, however, the variation in amount of activity in the cholesterol remained high.

The distribution of radioactivity between the column chromatographic fractions that were obtained from 4 separate incubations with mevalonate-2-C^{14} was: total, 17,030 cpm; steroid, 1360 cpm (8%); squalene, 5640 cpm (32%); lanosterol and companions, 3550 cpm (21%); and cholesterol and companions, 2120 cpm (12%).

Others have fractionated the steroids from similar incubations with labeled acetate (2, 3, 25). Accordingly, extensive purification of the steroid fraction was not investigated. The labeled steroids were chromatographed with traces of carrier testosterone (10–25 μg.) on thin layer plates of silica gel (12) or on alumina columns (2) with 1 mg. The mobility of the testosterone was slightly less than many of the companion steroids. The testosterone was eluted, acetylated (pyridine and acetic anhydride), and chromatographed again on silica gel plates with 3:1 benzene and ethyl acetate. The total activity of the testosterone acetate was 11–40% of the total activity in the original methanol extract.

TABLE VI

Distribution of Carbon Radioactivity in Chromatographic Fractions

The tissue from individual testes was suspended in 2.5 ml of buffer. Each flask contained 0.6 μc of acetate-1-C^{14} (720 nanoles) and glucose (125 μmoles). The tissue (about 70 mg. protein) was incubated for 3 hours under oxygen. "Steroid" is the crude fraction in the aqueous methanol; "sterol" is the crude fraction in pentane. Lanosterol is the combined lanosterol and companions; cholesterol is the combined cholesterol and companions. The values for the column chromatographic separation are the means and standard deviations of 11 separate incubations. The values for the thin layer separation are the means of 4 incubations.

Fraction	Column chromatography		Thin layer chromatography
	(cpm/flask)	(% of NSF[a])	(% of NSF[a])
Extracted NSF	13,300 ± 1870	100	100
"Steroid"	1280 ± 286	32	25.6 ± 1.85
"Sterol"	7680 ± 940	58	[b]
Squalene	1660 ± 303	43	33.6 ± 1.5
Lanosterol	2410 ± 1260	18	18.9 ± 1.7
Cholesterol	2280 ± 1200	17	15.9 ± 1.6
Recovered NSF	10,620	80	91.0

[a] NSF = nonsaponifiable fraction.

[b] Because small amounts of sterols are isolated with the steroids in aqueous methanol, the sterol that was recovered from thin layer chromatography of the crude steroids was added to the sterol in the pentane solution. Accordingly, no value for the crude sterol is reported.

Incorporation of Acetate into Testicular Sterols *in Vivo*

Because much of the activity in the nonsaponifiable fraction from incubation *in vitro* was associated with sterols other than

cholesterol, the appearance of similar labeled compounds from incubation *in vivo* was investigated. Acetate-1-C^{14} (25 μc) was injected into each of 9 rats. The rats were sacrificed 1, 2, and 3 hours after the injection. The average incorporation of label into the nonsaponifiable fraction of lipids from testicular tissue was low: 684, 881, and 2130 cpm per rat after 1, 2, and 3 hours, respectively. This represents a maximal incorporation of 0.0077% of the injected dose after 3 hours. Others have reported similar low incorporations of injected mevalonate by testicular tissue (26).

The crude extract of steroids contained 38% of the radioactivity after 1 hour, and 20 and 23% of the total activity after 2 and 3 hours, respectively. The average percentage of radioactivity in squalene (8), lanosterol (15), and cholesterol (31) remained relatively constant.

Incubation of Cell-Free Homogenates of Rat Testes

To determine whether cholesterol is an obligatory intermediate in the formation of testosterone from acetate, cell-free homogenates that incorporate acetate or mevalonate simultaneously into cholesterol and steroids are needed. Cell-free preparations of rabbit testis did not incorporate acetate, mevalonate, or butyrate into sterols and steroids (25).

Cell-free preparations of rat testicular tissue were incubated with various cofactors and additions. Labeled mevalonate or acetate was incubated with a homogenate (Waring blendor, 33% v/v) of testicular tissue in phosphate buffer (pH 7.4, 0.1 *M*). The homogenate was centrifuged before incubation (8000*g*, 30 minutes), and neutral solutions of the added materials were included alone and in combination. None of the additions stimulated the incorporation. The following compounds were investigated in various combinations at the indicated concentrations (m*M*): nicotinamide, 30; glutathione, 10, 30; ATP, 2, 12, NAD, 1, 5, 10; glucose, 50; NADPH, 2; NaF, 10, 30; and boiled liver extract of 20–100% of the homogenizing medium. Others have obtained stimulation of sterol biosynthesis by similar additions to cell-free homogenates of liver (27), ascites tumor cells (28), and preputial gland tumor (29). The complete lack of synthesis in the homogenate of testicular tissue in the absence of added compounds or liver extract was similar to ascites and preputial gland tumor cells.

Ascites and preputial gland tissue metabolize lanosterol (28, 29). Accordingly, labeled lanosterol was prepared from mevalonate-2-C^{14} by the method of Møller and Tchen (30). The labeled lanosterol was purified chromatographically (19), diluted with carrier, and suspended in a solution of Tween 80* in buffer (4 mg. Tween 80 per milliliter). Testicular tissue was homogenized in 2 volumes of phosphate buffer (pH 7.4, 0.1 *M*) that contained nicotinamide (0.03 *M*). The supernatant fraction (4 ml.; 10,000*g*, 30 minutes) was incubated for 3 hours at 37°C. under oxygen with 0.2 ml. of the lanosterol suspension (40 mμmoles, 5610 cpm) in each of 4 Erlenmeyer flasks that contained 2 mg. of NAD, and a center well and wick. Labeled carbon dioxide was collected as described previously (21). Normal homogenate yielded 10.7 ± 4.1 and 221 ± 12 cpm of C^{14} activity in carbon dioxide and C_{21}-sterols, respectively. Boiled homogenate liberated from 0–4 cpm of C^{14} activity in carbon dioxide. The yield of label in the C_{21} sterols was in slight excess (221/40.7 = 5.4).

Discussion

Others have suggested that mevalonic acid is not a precursor of steroids of the adrenal gland (31) and testicle (32). The findings in the present report are in agreement with the report of Rabinowitz (33) who demonstrated that mevalonic acid may be incorporated into the cholesterol and 17β-estradiol of testicular tissue. The studies with the dilution of acetate incorporation by mevalonate extend this conclusion; however, the dilution experiment will have to be verified when viable cell-free homogenates of rat testicular tissue are available.

Many investigators have demonstrated the incorporation of acetate into the crude digitonin precipitable sterols of testicular tissue (1, 2, 7). In all cases, the specific activity of the cholesterol digitonide decreased when the crude product was purified

with carrier cholesterol. The results of the present report indicate that the mixture of labeled products in the unfractionated digitonide contained not only labeled lanosterol and other digitonin precipitable sterols, but, in addition, substantial radioactivity from the nonsaponifiable fraction of lipids was associated with squalene and acyclic alcohols which are not precipitated by digitonin (23) but may be products that are related to the biosynthesis of sterols and steroids.

Currently, the variable ratio of radioactivity between the lanosterol and companions and the cholesterol and companions and the stimulation of the reactions that precede the formation of lanosterol are being investigated.

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CANKER SORES FROM ALLERGY TO WEAK ORGANIC ACIDS (CITRIC AND ACETIC)

Case Report and Clinical Study

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ALLERGY to foods manifests itself in different ways and can give rise to a host of clinical manifestations. The etiological relationship between the latter and the food allergy may be definite in some instances, as for example, the development of angioedema of the lip and/or asthma following the ingestion of egg by an egg-sensitive child. That egg is responsible is indicated not only by the clinical history, but by the finding of a positive skin test reaction to egg and by the presence of circulating antibodies (reagins) to egg. Whenever it is possible to obtain such confirmatory evidence, the etiological association between the offending food and the clinical condition is easy to prove. But, unfortunately, this is not true of all the clinical manifestations ascribed to food allergy, especially if the skin test reaction to the suspected agent is negative. Under those circumstances, proof of an etiological relationship must depend entirely upon the subjective clinical data supplied by the patient after the offending food or foods are eliminated while on special or trial diets. One explanation offered for the occurrence of a negative skin test reaction to the food despite positive clinical tests is that the allergy exists to a split product of the protein (for example, amino acids, as in the case previously reported by us[1]), or to an enzyme (for example, that present in raw fruits or melons[2]), or to some other as yet undiscovered agent which does not give positive skin test reactions.

Recently, we saw a patient in whom recurrent outbreaks of canker sores, ascribed at times to food allergy, seemed to be associated with an abnormal reaction or allergy to weak organic acids like citric and acetic, as indicated by the results of our clinical studies. Since we could find no record in the literature of the demonstration of a similar etiological association, it seemed worth while to report this case history and the results of our clinical investigations.

CASE REPORT

A man, aged 37 years, an Army veteran, was first seen in the Outpatient Allergy Clinic of the Veterans Administration in May, 1953, complaining of nasal blockage, asthma, and headaches. These symptoms had begun at the age of 5, following an attack of whooping cough, and had been recurring since then. Little improvement was noted either after a tonsillectomy and adenoidectomy at the age of 7 or after a submucous resection done at the age of 19.

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The patient enlisted in the United States Air Force in January, 1942. He noted some increase in symptoms during his period of training and while flying planes in the China-Burma-India theater, especially as the result of changes in temperature as well as from exposure to the inhalation of soft coal smoke in southern Illinois and to volcanic dust in the Aleutians. In 1950, while in Texas and because of the persistence of symptoms, he was sent to the Brooks General Hospital for allergy investigation. Positive skin test reactions were obtained to milk and to a few other foods; also to dust, feathers, pyrethrum, and ragweed. Because of the recurrence of more severe symptoms of asthma and migraine headaches, he was sent to the Lackland Base Hospital where desensitizing injections of dust and ragweed pollen extracts were given for more than a year. Following this treatment, he noted fewer nasal and bronchial symptoms during the ragweed season, but continued to have headaches when exposed to dust or after the ingestion of certain foods, such as milk or chocolate.

For many years, probably since early childhood, the patient had frequent canker sores in the mouth. These were sometimes associated with headaches and also with vague muscular pains and varying degrees of lassitude. The canker sores persisted throughout his army life and up to the time he was first seen in our clinic. Having had them for so many years, he never complained about them and regarded them as trivial.

The patient remained in the Air Force until May, 1952, when he was discharged. In September, 1953, he enrolled at the University of Pennsylvania, in the Liberal Arts College, majoring first in psychology and later in sociology. Because of the continuance of the asthma, rhinitis, and headaches, he applied for treatment at the Philadelphia Allergy Clinic of the Veterans Administration. Complete intracutaneous skin tests revealed significant positive reactions to house dust, ragweed, timothy, and plantain, with negative reactions to the commonly eaten foods including milk and chocolate.

Treatment was begun shortly afterward. It consisted of the elimination of test-positive allergens and of desensitization with extracts of timothy and ragweed pollens and of house dust. The desensitizing injections still are being continued. The patient reports definite improvement in his nasal symptoms and in his asthma. The latter now is quite mild and is readily controlled by symptomatic measures (for instance, Tedral). Despite the institution of allergy treatment, the patient continued to have recurrent canker sores and headaches; also frequent spells of general lassitude, vague pains, irritability, and inability to concentrate, which interfered considerably with his studies. Because of these symptoms, he consulted the psychiatrist at the Veterans Clinic, who considered the symptoms functional in origin.

In attempting to correlate his symptoms with the offending allergen, the patient reported that exposure to smoke or dust of any type would induce severe nasal blockage and wheezing; ingestion of chocolate, candy, beer, nuts, and seafood also would cause nasal symptoms and, at times, asthma. Of particular interest in relation to our subsequent studies was his observation that the eating of chocolate (to which he had a negative intracutaneous skin test reaction) would provoke an outbreak of "sore spots" or canker sores in the mouth associated at times with "itchy bumps" on the scalp. The patient was quite accustomed to these canker sores, and for years had attributed them to the ingestion of sugars, sweets, "acid foods," or certain alcoholic drinks. The effect of the latter was verified by one episode recalled by the patient which took place during his tour of duty in Asia and which is of special interest in view of our subsequent clinical studies. Upon returning to the home base after a long tour of flying, the flight medical officer usually advised the pilots to take a few drinks to ease the tension and relieve the customary headaches which followed most flights. When the patient tried this, he usually developed migraine headaches, asthma, and canker sores. These symptoms at times were so severe that he could not report for duty, and he therefore seldom drank. He recalled that whenever he took mixed drinks containing fruit juices, he invariably developed symptoms; on the other hand, when he drank straight liquor, he might develop nausea associated with a mild "hangover," but would seldom get asthma, nasal blockage, or canker sores. The reasons for this difference will be evident later.

One of us (L. T.) saw the patient for the first time in May, 1954, in consultation. In reviewing the history with the patient, it seemed likely that the canker sores followed the ingestion of certain foods, especially grapes or anything made from grapes (for example, grape juice, jelly, wine, brandy); also, after eating chocolates and certain kinds of candies and carbonated beverages. To verify this possibility, we undertook further clinical studies and were fortunate in having the complete cooperation and exceptional assistance of a highly intelligent patient.

Clinical Studies.—A survey of the ingestants known clinically to produce his mouth symptoms indicated that one common factor nearly always was present, namely, citric acid. This substance was present either as a naturally occurring organic acid, as in citrus fruit, or it was used as an ingredient in the manufacture of commercially prepared products, as in carbonated beverages. The possibility that an organic acid such as citric acid could act as an allergen or could act as a mucous membrane irritant had never occurred to us and has not been noted in the allergy literature. Our attention to citric acid was drawn especially by some observations of this patient after a rather accidental experience.

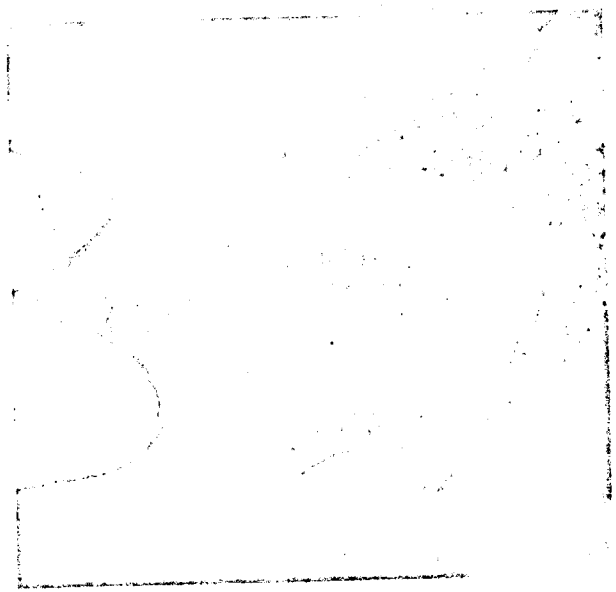

Fig. 1.

Because of the restriction in diet, especially of milk, the patient felt weak and began to eat candy bars. This only seemed to make him worse. It induced heartburn, epigastric discomfort, gas, headache, canker sores, and general lassitude, and made concentration difficult. He attributed this to the chocolate and excess sugar and so tried other candies.

He began eating Necco wafers, especially while in class, and would take one of the wafers from the package in his pocket and slip it into his mouth without seeing it. He reported that certain wafers seemed to cause a puckering or itching sensation in the mouth and peculiar discomfort in the stomach. Upon further investigation, he noted that this would happen after eating yellow, green, or orange wafers which apparently contained citric acid, but not from the white, cinnamon, or even the chocolate-flavored ones, presumably those without citric acid. To eliminate the possibility that sugar was responsible, he ate pure cane sugar cubes and also rock candy without difficulty. Since he could take the chocolate wafer without trouble, he tried eating the pure chocolate which

is used by confectioners or bakers in preparing candy or pastry. This caused no symptoms, whereas most of the commercial chocolate bars, which according to the labels contained citric acid, induced canker sores.

This seemed to indicate that the patient was not allergic to chocolate itself but to substances combined with it. As a result of these experiences, the patient became quite adept at determining whether anything he ate or drank contained citric acid. Thus, for example, he found that he could not tolerate certain cough drops or troches (such as the red-colored Smith Brothers), whereas others were all right. This also was true of certain beverages; for example, he could drink unlimited amounts of Hires Root Beer (which, according to the label, does not contain citric acid) without symptoms, whereas one Coca-Cola or even club soda (both of which do contain citric acid) induced canker sores. As a result of these experiences, the patient became label conscious, inspecting all labels for citric acid and avoiding foods containing it. By strictly avoiding all substances containing citric acid, the patient could remain relatively free of canker sores for long periods and also could induce the sores at will by eating citric acid-containing foods.

Because of our suspicion that citric acid was the culprit, we coincidently began a series of investigations designed to determine whether our clinical suspicions could be verified. Since substances like citric acid do not give positive skin test reactions even in sensitive patients, we felt that there was a better chance of verification if we used a direct mucosal contact test. Accordingly, we had the patient put a small crystal of citric acid (contained in sour salt) into the oral cavity in the space formed by the lower lip and the lower anterior alveolar surface. Within a few hours, there was a reddening and edema at this area. By the next day a white, raised patch 12 to 18 mm. in diameter was present, with two or three papular elevations around its border. The area sloughed away, leaving a craterlike ulcer with slightly raised edges (Fig. 1). Following the healing of this ulcer, which required about ten days, the patient then repeated the test in a different area, leaving the crystal in contact for fifteen seconds. Again the same chain of events occurred at the contact point, but a smaller area was involved. By later tests, it was found that even momentary contact of a citric acid crystal on the oral membranes was sufficient to produce canker sores. Control tests with potassium citrate crystals were negative, and tests with citric acid crystals upon members of the patient's family and many other individuals were negative. We then tried other related substances, or those containing citrate, with results shown in Table I. All the substances tested were in the form of crystals except magnesium citrate, which was in liquid form. The latter was tested by moistening a pledget of cotton with the solution and placing it in the oral cavity in the same manner as the crystals.

Table I. Results of Mucosal Contact Tests With Citric Acid and Related Substances

Substance	Result of Contact Test
Citric acid (sour salt)	Strongly positive
Potassium citrate	Negative
Magnesium citrate (solution)	Negative
Alka-Seltzer (citric acid, aspirin, sodium bicarbonate)	Moderately positive
Aspirin	Negative
Sodium bicarbonate	Negative
Bromo-Seltzer (citric acid, acetanilid, caffeine, sodium bicarbonate, sodium bromide)	Negative

It will be observed from Table I that all the compounds containing free citric acid gave positive mucosal tests with the exception of Bromo-Seltzer. The negative test to the latter perhaps can be explained by the fact that the citric acid in Bromo-Seltzer, when dissolved, quickly changes to sodium citrate. It is noteworthy that when the patient was

testing it, he noticed that when he placed the crystal against the oral mucosa it dissolved quickly in comparison with Alka-Seltzer crystals which remained in solid form much longer.

While it seemed evident from the results of our contact tests that citric acid was responsible for the canker sores in the clinical experiments, a question arose as to whether it was a matter of pH; also, even though the resultant ulcers were seen by one or both of us after the completion of some of the tests, observations of the patient himself had to be taken in reporting the results of most tests. To check this, blind mucosal tests were done by the patient with two solutions prepared by Dr. Hamilton* and marked "A" and "B." The patient was instructed to test the solutions by means of pledgets of cotton moistened in the solution and applied to the oral mucosa for a period of three minutes in the first test, and fifteen minutes in the later one. Different mucosal areas were used each time and sufficient time was allowed to elapse to permit the ensuing reaction to diminish. Solution "A" produced negative results both times. Solution "B", after three minutes, produced a definite positive reaction in the form of a red patch; after fifteen minutes, a much larger area of redness was noted, although no ulcer formed, probably because of the weak concentration used. Following the completion of these tests, we were told by Dr. Hamilton that Solution "A" was a weak solution of HCl (tenth normal), whereas Solution "B" was a weak solution (about 2.5 per cent) of citric acid in corresponding pH. These experiments, therefore, seemed to confirm the previous observations that citric acid was the specific offending agent, rather than the "acidity" or the irritative qualities of an acid substance.

TABLE II. RESULTS OF MUCOSAL CONTACT TESTS WITH ORGANIC ACIDS

SUBSTANCE	RESULT OF CONTACT TEST
Tartaric acid	Mildly positive
Acetic acid (apple vinegar)	Strongly positive
Lactic acid	Mildly positive
Uric acid	Negative
Ascorbic acid	Negative
Nicotinic acid	Negative
Benzoic acid (1%)	Negative

The question next arose as to whether other organic acids might act in a similar fashion to citric acid. After consultation with Dr. Hamilton, a series of mucosal tests, done either with the pure crystals or with a solution soaked in a cotton pledget, were carried out by the patient with those organic acids most frequently found in the human diet. The results of these tests are summarized in Table II.

The marked positive mucosal reaction to the application of acetic acid was surprising. But, at the same time, it explained why the patient had mouth ulcers and gastrointestinal symptoms after eating foods containing vinegar; thus, for example, the patient reported that he would develop a typical right sided migraine headache as well as canker sores and itching of the skin after eating foods containing vinegar, such as cole slaw, potato salad, or beef cooked in vinegar, whereas he could eat plain beef with impunity. These symptoms were similar to those he noted after taking foods with excess citric acid (for example, fruit juices) but seemed more intense perhaps because he took larger amounts.

Another question presenting itself for further investigation was whether the organic acids, like citric acid, acted by direct contact with the buccal mucosa or after ingestion or by both means. To determine this, the patient took a 3 grain (0.2 Gm.) capsule (washed down) of citric acid shortly after breakfast. He had no immediate reaction; but several hours later noted a gnawing pain in the epigastrium, mild diarrhea, and

*The authors are greatly indebted to Robert H. Hamilton, M.D., Professor of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania, for his invaluable advice and assistance in carrying out some of the biochemical studies and for some of the material employed in the tests.

slightly irritated tongue. A month later (December, 1954), he took a 5 grain (0.3 Gm.) capsule of citric acid after each of his three meals for two days, but noticed no outward effects. Despite these results, the patient observed that ingestion of foods containing excess citric acid (fruit juices or wine) produced not only canker sores but at times inflamed furuncle-like lesions usually in the nares and less commonly in the lower lid (resembling a stye), mostly on the right side. This would seem to indicate that the organic acids act not only after direct contact but also after getting into the stomach and intestines, depending upon the amount ingested and the amount absorbed into the circulation.

Further clinical trials were made with other foods, especially fresh fruits, to determine whether they would induce the formation of canker sores. As a result of these tests, it was determined that canker sores could be provoked not only by commercial foods or drugs containing citric or acetic acids but also after the ingestion of the following foods: oranges, grapes, dried apricots, raw and canned peaches, pineapple, wines especially Chianti, brandies, beer, and most mixed alcoholic drinks. On the other hand, he determined that he could eat with impunity the following: raw or cooked apple, raw or canned pears, canteloupe, watermelon, honeydew melon, Persian melon, and raw celery; however, canned celery juice caused a puckering sensation in his mouth, perhaps because of the added preservative. While most of the reacting foods induced canker sore formation, this was not true of all of them. Thus, ingestion of milk or cheese precipitated headache, malaise, and wheezing, but no canker sores.

Following the diagnostic study and especially after extensive clinical trials which revealed the offending agents, the patient eliminated as far as possible all offending foods as well as those containing citric acids or acetic acids. As a consequence, he has noticed definite lessening of his allergic symptoms except when he inadvertently or consciously is indiscreet in his diet; if so, he can easily detect the cause of his symptoms in retrospect; or if it is a canned food, he later finds that the food contained citric acid of which he previously was unaware. He also has noted definite improvement in his general physical and mental condition, especially in his power of concentration and in a lessening of his previous irritability. The latter condition was the basis for his periodic visits to the Clinic psychiatrist for two years, but he noted no beneficial effect upon his symptoms until after the offending foods were eliminated. He now sees the Clinic psychotherapeutist (psychologist) periodically for assistance in his school problems.

COMMENT

The clinical study of this patient has revealed many interesting features which might find application to other similar patients. In the first place, it was demonstrated that the patient had a definitely abnormal reaction or allergy to citric acid and to foods or drugs containing it. This reaction took the form of canker sores proved by direct mucosal contact tests with citric acid in this patient, whereas the same agent in other control patients gave negative reactions. Similar abnormal reactions were observed from other organic acids, notably acetic acid. One might question the use of the term *allergy* to designate a reaction to nonantigenic substances like citric or acetic acids, but similar abnormal reactions designated *allergic* occur in relation to various nonprotein agents (for instance, drugs) which likewise give neither a positive skin test reaction nor demonstrable antibody. Regardless of the designation, our demonstration in this patient, apparently for the first time, of an allergy to weak organic acids is of great importance in food allergy. It is another link in the chain of evidence indicating that allergic reactions to foods need not necessarily occur in relation to the protein component but may take place in relation to the split products like peptones and amino acids, or

preformed weak organic acids, and possibly to organic acids formed from substances in food other than proteins. Such patients may have negative skin test reactions to the parent food and yet possess an allergy (of the nonatopic type) to one of its products. This helps to explain those instances where the patient has a clinical allergy to a food and yet gives a negative skin test reaction to its extract. Another interesting feature in this patient is the fact that we were able to prove by direct mucosal tests that the canker sores were caused by the abnormal reaction to the weak organic acids. Similar testing with citric acid crystals and/or a weak solution of acetic acid might be carried out in those allergic patients in whom food allergy is suspected but the skin test reactions are negative, as for example in patients with canker sores.

Despite the clinical demonstration in this patient that contact with weak organic acids induced the formation of canker sores, the manner by which this is brought about raises many unexplained questions. First is the matter of specificity. Thus, canker sores were produced in this patient not only by citric acid but also by acetic acid and, to a mild degree, by tartaric and lactic acids. Likewise, it is not easy to explain, for example, why the canker sores were induced by contact with citric acid but not from sodium citrate. The evidence needed to answer these questions involve such matters as pH, concentration, solubility, or the buffer powers of organic acids compared with that of weak HCl and can be furnished only by further experimental investigation which is now in progress.

The fact that canker sores in this patient were proved to be due to allergy to citric acid and acetic acids is of interest also because of the popular custom of physicians and patients to attribute these canker sores to an excessive ingestion of "acid foods," especially fruit juices. Suspicion of an etiological relationship could not always be verified, except for a lessened tendency for the ulcers to develop when the suspected foods were eliminated. Our investigations furnish greater support to what previously seemed like mere suspicion and indicates that "acid foods" actually can have a causal relationship. They also explain the tendency of the patient to get canker sores when he drank mixed drinks containing fruit juices and not from straight liquors.

Finally, the clinical history in this patient demonstrates that symptoms regarded as functional or psychosomatic sometimes may be due to an unsuspected or unproved food allergy. Thus, in the case herein reported, symptoms of "toxemia," including headache, muscular neuralgic pains, lassitude, irritability, and inability to concentrate, improved considerably after the elimination of the offending foods, including those containing citric acid and acetic acid, and reappeared after their readdition. This is not new, for Rowe,[3] many years ago, included these symptoms in a syndrome designated by him as "allergic toxemia," but its existence in some patients sometimes is forgotten in our present emphasis upon functional causes.

SUMMARY

A report has been made of a male patient, aged 37, treated for nasal allergy, asthma, and migraine who for years was subject to frequent outbreaks

of oral canker sores. Clinical investigation indicated that the latter usually followed the ingestion of foods, drugs, or carbonated beverages containing citric acid. Direct application of citric acid crystals to the oral mucosa repeatedly reproduced the ulcer in the patient, but not in the controls. Mucous membrane contact tests with substances containing citric acid gave similar positive results; likewise, tests with other weak organic acids contained in foods gave positive reactions to some of these, especially to acetic acid. Avoidance of foods containing the positive reactors was followed by marked relief not only of the ulcers but also of the general "toxic" symptoms previously regarded as functional. This demonstration of possible allergic reactions to weak organic acids in foods provides additional explanation for the existence of clinical allergy to certain foods in some patients despite the presence of a negative skin test reaction.

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CHEMICALS AND PLASTICS
RESEARCH AND DEVELOPMENT

November 8, 1973

Dr. H. Sassoon
Tracor-Jitco, Inc.
1300 East Gude Drive
Rockville, Maryland 20851

Dear Dr. Sassoon:

As per our telephone conversations, I am sending you copies of Carbide's Specifications, and Specifications Methods of Analysis for Acetic Acid (FCC Grade), Sodium Acetate, and Sodium Diacetate (FCC Grade). I trust that by now Celanese has sent you theirs. Your suggestion about your contacting Mallinkrodt is an excellent one because of the possible legal ramifications that we spoke of, which could conceivably arise should I contact them.

If after reviewing our material you feel that it is not sufficient for your purposes, please do not hesitate to let me know.

Very truly yours,

Bill Scott

Bill Scott

/slm
Attachments

SODIUM DIACETATE - FOOD CHEMICALS CODEX GRADE

		Specification Limits	Method
1	Sodium acetate	58.0 to 60.0 per cent by weight	1B-32A4-1
2	Acetic acid	39.0 to 41.0 per cent by weight	1B-32A4-1
3	Water	2.0 per cent by weight, maximum	1B-32A4-1
4	Acetic acid, sodium acetate, and water	99.7 per cent by weight, minimum, as total	1B-32A4-1
5	Formic acid, formates, and other oxidizable impurities	0.2 per cent by weight, maximum, as formic acid	1B-32A4-1
6	Arsenic	3 ppm, maximum as As	1B-32A4-1
7	Heavy metals	10 ppm, maximum, as Pb	1B-32A4-1
8	Iron	10 ppm, maximum, as Fe	1B-32A4-1
9	Appearance of 10 per cent solution in water	Shall be clean and not more than 40 platinum-cobalt in color	1B-32A4-1
10	Appearance	White crystalline powder when packaged. (Material has a tendency to cake on storage.)	1B-32A4-1
	Reference	Food Chemicals Codex, 1966 Edition	

RESEARCH AND DEVELOPMENT DEPARTMENT
TECHNICAL CENTER
South Charleston, West Virginia

whn

SODIUM DIACETATE

1 SODIUM ACETATE

a) Crystal violet indicator; Reagent number 10T44.2, 0.1 per cent (wt/volume) in acetic acid. Dissolve 0.1 gm of crystal violet in 100 ml of glacial acetic acid.

b) Standard 0.1 N perchloric acid in acetic acid Transfer 8 ml of 70-72 per cent perchloric acid to a 1-liter volumetric flask containing 500 ml of glacial acetic acid. Swirl to effect solution and dilute to the mark with additional glacial acetic acid. Allow the reagent to stand overnight before standardizing.

c) Procedure Transfer 0.28 to 0.32 gm of sample weighed to the nearest 0.1 mg to each of two glass-stoppered Erlenmeyer flasks containing exactly 50 ml of glacial acetic acid. Swirl to effect complete solution. The sample will dissolve more readily if the glass-stoppered flask is held in hot water and swirled. Reserve additional acetic acid for a blank determination.

d) Cool the flask and contents, add exactly 2 drops of crystal violet indicator, and titrate with the standard perchloric acid in acetic acid to the first permanent blue-green color.

e) Calculation

$$\frac{(A-B)N \times 8.204}{\text{gm sample}} = \text{sodium acetate, per cent by weight}$$

A = ml of N normal perchloric acid in acetic acid required for the sample

B = ml of N normal perchloric acid in acetic acid required for the blank

2 ACETIC ACID

a) Transfer 0.5 gm of the sample weighed to the nearest 0.1 mg to a 250-ml Erlenmeyer flask containing 50 ml of distilled water.

b) Add 4 to 6 drops of 1.0 per cent alcoholic phenolphthalein indicator.

c) Titrate with standard 0.1 N sodium hydroxide to a pink end point permanent for at least 15 seconds.

d) Calculation

$$\frac{AN \times 6.005}{\text{gm sample}} = \text{acetic acid, per cent by weight}$$

A = ml of N normal sodium hydroxide required for the sample

WATER

a) Stock solution of acetic acid - SO_2 Dissolve 135 gm of anhydrous SO_2 in 900 ml of acetic acid.

b) Acetic acid - SO_2 reagent Dissolve 50 ml of the stock solution of acetic acid - SO_2 to one liter with acetic acid.

c) Procedure Transfer 50 ml of the acetic acid - SO_2 mixture into each of three glass-stoppered Erlenmeyer flasks.

d) From a buret add SO_2-I_2 reagent (F = approx. 0.2) to each flask until a permanent light reddish brown color is obtained. Match the colors of the solutions in transmitted light by the dropwise addition of reagent.

e) Reserve one of the flasks as a blank.

f) Into each of the other flasks introduce the following specified sample size weighed to nearest 0.1 mg.

Grade	Sample wt, gm
Sodium Diacetate - Technical Grade	1.8 to 2.2
Sodium Diacetate - Anhydrous Grade	14.9 to 15.1

g) Immediately titrate with the SO_2-I_2 reagent until the light reddish brown color persists using the blank as a guide to the proper color at the end point.

$$\frac{A \times F}{\text{gm sample}} = \text{water, per cent by weight}$$

A = ml of SO_2-I_2 required for the sample
F = factor, gm of water equivalent to 100 ml of reagent

4 ACETIC ACID, SODIUM ACETATE, AND WATER

a) Calculation

A + B + C = acetic acid, sodium acetate and water, % by wt

A = acetic acid, per cent by weight, paragraph 2d
B = sodium acetate, per cent by weight, paragraph 1e
C = water, per cent by weight, paragraph 3g

5 FORMIC ACID, FORMATES, AND OTHER OXIDIZABLE IMPURITIES

a) Transfer 1.0 gm of sample weighed to the nearest 0.1 mg to a suitable flask or beaker containing approximately 50 ml of distilled water. Add 10 ml of 10 per cent sulfuric acid and heat the solution to approximately 80 to 90°C.

b) Titrate the hot solution with 0.1 N potassium permanganate solution until a faint permanganate color persists. Reheat the solution, if necessary, to maintain the temperature at approximately 80 to 90°C.

c) Calculation

$$\frac{AN \times 2.301}{\text{gm sample}} = \text{oxidizable impurities, \% by wt as formic acid}$$

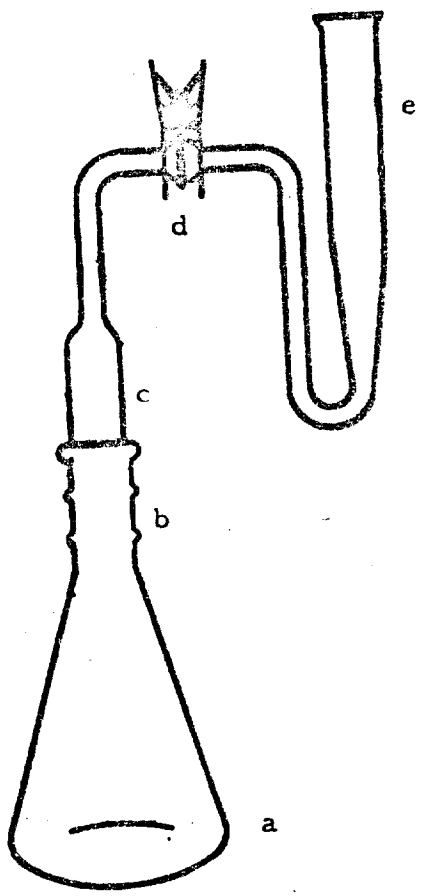
A = ml of N normal potassium permanganate required for the titration

6 ARSENIC

NOTE! All glassware for this determination must be meticulously clean. Best results are obtained if this glassware is retained exclusively for use in the arsenic determination.

a) Apparatus Assemble the apparatus as shown in Figure 1.

APPARATUS FOR ARSENIC TEST



a - 125-ml generator flask
b - 24/40 standard taper joint
c - scrubber tube
d - ball and socket joint
e - absorber tube

FIGURE 1

b) Standard arsenic solution Accurately weigh 0.132 gm of arsenic trioxide that has been finely pulverized and dried for 24 hours over a suitable desiccant, and dissolve it in 5 ml of 20 per cent aqueous sodium hydroxide. Neutralize the solution with 10 per cent sulfuric acid solution and add 10 ml in excess. Quantitatively transfer this solution to a 1000-ml volumetric flask, dilute to the mark with recently boiled distilled water, and mix well. By means of a suitable pipet, transfer 10 ml of this solution to a second 1000-ml volumetric flask. Add 10 ml of 10 per cent sulfuric acid, dilute to the mark with distilled water and mix well. This final dilution which contains 1 μg of arsenic (As) per ml, must be prepared fresh every three days.

c) Silver diethyldithiocarbamate solution Dissolve 1 gm of silver diethyldithiocarbamate, $(C_2H_5)NCSSAg$, in 200 ml of reagent grade pyridine. Store this reagent in a brown-glass bottle and prepare fresh every month.

d) Stannous chloride solution Dissolve 40 gm of stannous chloride dihydrate ($SnCl_2 \cdot 2H_2O$) in 100 ml of concentrated hydrochloric acid. Prepare this reagent fresh every three months.

e) Lead acetate-impregnated cotton Soak cotton in a saturated solution of lead acetate, squeeze out the excess solution, and dry in a vacuum at room temperature.

f) Sample solution Transfer 1.00 gm of sample to the generating flask (Figure 1) and carefully add 5 ml of concentrated nitric acid. Add one or two small glass beads and cautiously add 2 ml of concentrated sulfuric acid in small increments. Place a stemless funnel in the neck of the flask and place the flask on an electric hot plate maintained at a moderate temperature. Regulate the heating in a manner to prevent excessive foaming, and rotate the flask occasionally to prevent the sample from caking. Maintain oxidizing conditions at all times during the digestion by adding small quantities of nitric acid whenever the mixture turns brown or darkens. Record the total volume of nitric acid used in this procedure. Continue the digestion until the organic matter is destroyed, fumes of sulfuric acid are copiously evolved, and the solution becomes colorless or retains only a light straw color. Cool the flask, cautiously add 10 ml of distilled water, and again evaporate to strong fuming, repeating if necessary to remove all of the nitric acid. Cool, dissolve the residual liquid in about 10 ml of distilled water, wash the funnel and sides of the flask with a few ml of water, and dilute to 35 ± 2 ml.

g) Procedure Add 20 gm of dilute sulfuric acid (1·5 solution) to the flask.

h) By means of suitable transfer pipets, add 2 ml of 15 per cent aqueous potassium iodide and 0.5 ml of the stannous chloride solution. Swirl gently to mix and allow the solution to stand for 30 minutes at room temperature.

i) Pack the scrubber tube of the apparatus with two plugs of the lead acetate-impregnated cotton in a manner to insure that all evolved vapors will be contacted. Lubricate the joints b and d with a suitable stopcock grease and connect the scrubber unit to the absorber tube c.

j) By means of a suitable transfer pipet, introduce 3 ml of the silver diethyldithiocarbamate solution to the absorber tube.

k) Add 3.0 gm of granular zinc (No. 30 powder) to the mixture in the generator flask and immediately insert the standard-taper joint in the flask.

l) Allow the evolution of hydrogen and the color development to proceed at room temperature (not below 25°C) for 45 minutes, or until gas evolution is complete, gently swirling the flask at 10-minute intervals. Take care to prevent the absorber solution from being drawn back into the scrubber tube when the gas evolution is complete.

m) Disconnect the absorber tube from the generator and scrubber units and transfer the silver diethyldithiocarbamate solution to a 1-cm Beckman Model B Spectrophotometer cell.

n) Determine the absorbance of the solution at a wavelength of 525 mμ, based on a reading of zero for a blank of the original silver diethyldithiocarbamate solution. The absorbance must be less than that obtained from 3.0 ml of standard arsenic solution when treated as described below, indicating less than 3 ppm arsenic (As).

o) Standard color solution Perform the oxidation procedure described in paragraph f, using 3.0 ml of the standard arsenic solution in place of the sample. Evaporate the 3.0 ml to dryness and oxidize with 2 ml of concentrated sulfuric acid plus the total volume of nitric acid used in paragraph f. After oxidation is complete, dilute to 35 ± 2 ml with distilled water.

p) Develop and measure the color absorbance produced from this solution, following the procedure described in paragraphs g through n.

q) This standard color must be prepared for each freshly-prepared standard arsenic solution, paragraph b.

7 HEAVY METALS

CAUTION! Use only glass containers free of soluble lead salts.

a) Lead stock solution Transfer 0.1598 gm of reagent grade lead nitrate, $Pb(NO_3)_2$, to a 1000-ml glass-stoppered volumetric flask containing 100 ml of distilled water and one ml of concentrated c.p. nitric acid. Swirl to effect solution, dilute to the mark with distilled water, stopper the flask, and mix thoroughly. Reserve this solution as the lead stock solution.

b) Standard lead solution Introduce 10 ml of the lead stock solution, paragraph a, into a 100-ml glass-stoppered volumetric flask by means of a suitable transfer pipet. Dilute to the mark with distilled water, stopper the flask, and mix thoroughly. This standard solution must be prepared fresh daily. A 1.0-ml portion of this solution is equivalent to 10 ppm of lead when using a 1.0 gm sample.

c) Procedure Introduce 1.0 gm of the sample weighed to the nearest 0.01 gm into one of two, 50-ml matched Nessler tubes. Add 1.0 ml of 1 N hydrochloric acid and sufficient distilled water to make a 25-ml volume.

d) Into the second tube pipet 1.0 ml of the standard lead solution and add 2 ml of diluted acetic acid (5.7 to 6.3 gm of acetic acid in 100 ml of distilled water) and sufficient distilled water to make a 25-ml volume. Reserve this solution as a standard.

e) To each tube add 10 ml of a saturated aqueous solution of hydrogen sulfide, stopper with a clean rubber stopper, mix thoroughly, and allow to stand for 10 minutes at room temperature.

f) Compare the color of the sample with that of the standard by viewing down through the tubes against a white surface. If the color of the sample is darker than that of the standard, the heavy metals content is in excess of 10 ppm.

IRON

a) Standard iron solution Dissolve 0.702 gm of ferrous ammonium sulfate, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, in 10 ml of 10 per cent aqueous sulfuric acid and dilute to 1000 ml with distilled water. Mix thoroughly. One ml of this solution contains 0.1 mg Fe.

b) Procedure Transfer 4.0 gm of the sample, weighed to the nearest 0.1 mg, to a suitable beaker containing 20 ml of distilled water and 15 ml of concentrated c.p. hydrochloric acid. Mix thoroughly to effect complete solution.

c) Transfer this solution to a 50-ml glass-stoppered mixing cylinder. Wash the beaker once with 5 ml of distilled water and add the washing to the mixing cylinder.

d) To a second 50-ml glass-stoppered mixing cylinder, add 25 ml of distilled water and 15 ml of concentrated c.p. hydrochloric acid. Reserve as the blank.

e) To each cylinder add one or two crystals of ammonium persulfate and 5 ml of a 10 per cent aqueous solution of potassium thiocyanate. Mix the contents of each cylinder thoroughly.

f) Transfer the contents of each cylinder to respective 23-ml cylindrical absorption cells and determine the absorbance of the sample solution on the Fisher Electrophotometer, using the 525B filter.

g) From a previously prepared calibration curve, read the ppm iron corresponding to the absorbance.

h) Calibration To respective 50-ml glass-stoppered mixing cylinders containing 20 ml of distilled water and 2 ml of concentrated c.p. hydrochloric acid, add 0.08, 0.10, 0.20, and 0.40 ml of the iron standard. Reserve a fifth cylinder as the blank. Dilute the contents of each cylinder to 40 ml with distilled water.

i) To each cylinder add one or two crystals of ammonium persulfate and 5 ml of 10 per cent aqueous potassium thiocyanate and mix the contents of each cylinder thoroughly.

j) Transfer the contents of each cylinder to respective 23-ml cylindrical absorption cells and measure the absorbance of each standard against the blank, using the Fisher Electrophotometer and the 525B filter.

k) Plot the ppm iron (based on a 4-gm sample) against the absorbance readings.

9 APPEARANCE OF 10 PER CENT SOLUTION IN WATER

a) Dissolve 10 gm of sample, weighed to the nearest 0.01 gm, in sufficient distilled water to give a final volume of 100 ml.

b) Examine the solution for any haze or turbidity.

c) Transfer the sample solution to a 100-ml tall-form Nessler tube.

d) Compare the color of the solution with those of a series of platinum-cobalt standards of equal volume in matched 100-ml Nessler tubes. View vertically down through the tubes against a white background.

e) Report the exact color of the sample solution in platinum-cobalt units.

10 APPEARANCE

a) Observe a portion of the sample in transmitted light. The sample shall be a white crystalline powder.

RESEARCH AND DEVELOPMENT DEPARTMENT
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South Charleston, West Virginia

rlp

"NIACET" SODIUM ACETATE, ANHYDROUS - FOOD CHEMICALS CODEX GRADE

		Specification Limits	Method
1	Sodium acetate	99.0 to 101.0 per cent by weight	1B-32A1-1
2	Water and/or acetic acid	0.8 per cent by weight, maximum	1B-32A1-1
3	Loss on drying	2.0 per cent by weight, maximum	1B-32A1-62
4	Alkalinity	0.2 per cent by weight, maximum, as NaOH	1B-32A1-62
5	Halides	0.3 per cent by weight, maximum, as NaCl	1B-32A1-1
6	Carbonates	Trace, as Na_2CO_3	1B-32A1-1
7	Iron	0.005 per cent by weight, maximum, as Fe	1B-32A1-1
8	Arsenic	3 ppm, maximum	1B-32A1-62
9	Heavy metals	10 ppm, maximum, as Pb	1B-32A1-62
10	Potassium compounds	None by test	1B-32A1-62
11	pH of a 10 per cent solution	6.5 to 8.5	1B-32A1-1
12	Appearance	White powder	1B-32A1-1
	Reference	Food Chemicals Codex, Second Edition, 1972	

"NIACET" SODIUM ACETATE, ANHYDROUS - FOOD CHEMICALS CODEX GRADE

1 LOSS ON DRYING

a) Introduce approximately 1 to 2 gm of sample into a tared glass-stoppered 30 x 70-mm weighing bottle. By gentle sidewise shaking, distribute the sample as evenly as possible over the bottom of the bottle. Stopper and reweigh the bottle, and obtain the sample weight by difference to the nearest 0.1 mg.

b) Place the weighing bottle in a vacuum oven maintained at 110°C. Remove the stopper and leave it also in the oven.

c) Slowly reduce the pressure in the oven using a suitable oil vacuum pump to the lowest practicable pressure.

d) Allow the sample to dry under these conditions for 2.5 hours.

e) At the end of the 2.5 hours release the vacuum slowly until atmospheric pressure is attained in the oven.

f) Replace the stopper in the bottle and allow the bottle to cool to room temperature in a suitable desiccator.

g) Reweigh the bottle and contents to the nearest 0.1 mg and obtain the weight of the dried sample by difference.

h) Calculation

$$\frac{(B-A) \times 100}{B} = \text{loss on drying, \% by weight}$$

A = weight of dried sample, paragraph g
B = weight of original sample, paragraph a

2 ALKALINITY

a) Introduce 2.0 gm of the sample weighed to the nearest 0.1 mg into a suitable Erlenmeyer flask.

b) Add 20 ml of distilled water and swirl the contents to effect complete solution.

c) Add 3 drops of 1.0 per cent alcoholic phenolphthalein indicator to the flask.

d) From a 10-ml buret add dropwise standard 0.1 N sulfuric acid just to the disappearance of the pink color. Not more than 1.0 ml of the standard 0.1 N sulfuric acid shall be required for the neutralization. This is equivalent to 0.2 per cent by weight as NaOH.

3 ARSENIC

NOTE! All glassware for this determination must be meticulously clean. Best results are obtained if this glassware is retained exclusively for use in the arsenic determination.

a) Apparatus Assemble the apparatus as shown in Figure 1.

APPARATUS FOR ARSENIC TEST

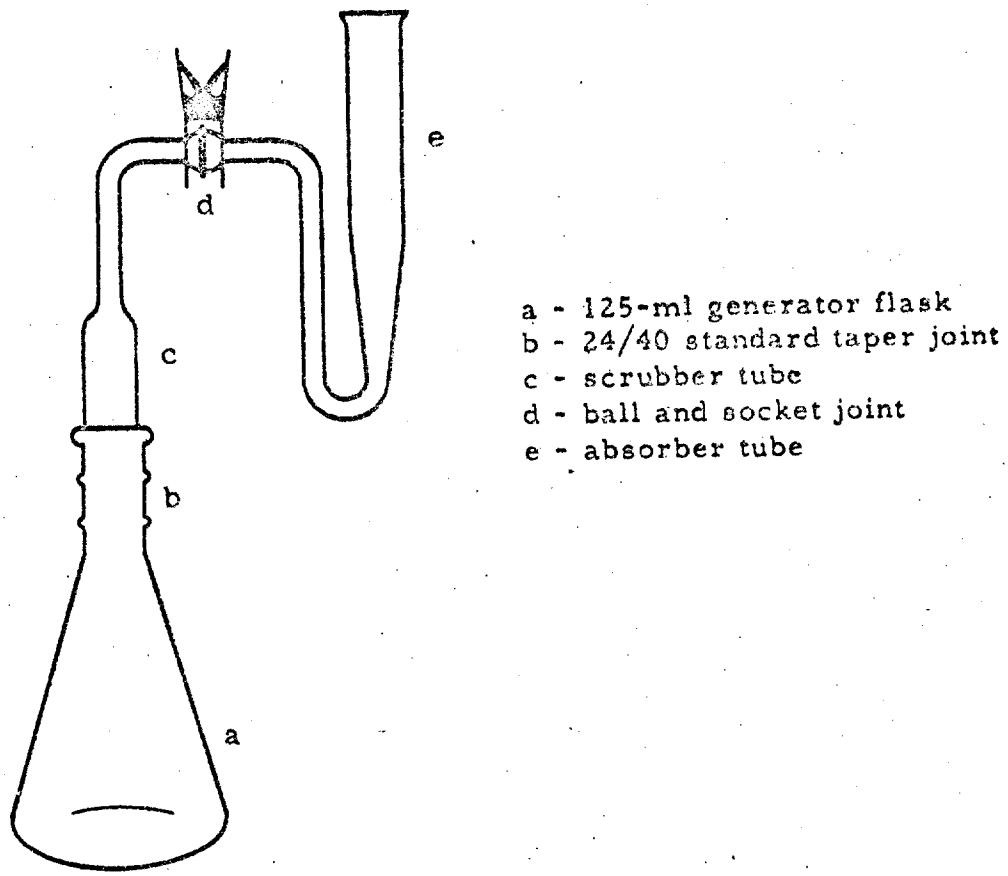

FIGURE 1

b) Standard arsenic solution Accurately weigh 0.132 gm of arsenic trioxide that has been finely pulverized and dried for 24 hours over a suitable desiccant, and dissolve it in 5 ml of 20 per cent aqueous sodium hydroxide. Neutralize the solution with 10 per cent sulfuric acid solution and add 10 ml in excess. Quantitatively transfer this solution to a 1000-ml volumetric flask, dilute to the mark with recently boiled distilled water, and mix well. By means of a suitable pipet, transfer 10 ml of this solution to a second 1000-ml volumetric flask. Add 10 ml of 10 per cent sulfuric acid, dilute to the mark with distilled water and mix well. This final dilution, which contains 1 μg of arsenic per ml, must be prepared fresh every three days.

c) Silver diethyldithiocarbamate solution Dissolve 1 gm of silver diethyldithiocarbamate, $(C_2H_5)NCSSAg$, in 200 ml of reagent grade pyridine. Store this reagent in a brown-glass bottle and prepare fresh every month.

d) Stannous chloride solution Dissolve 40 gm of stannous chloride dihydrate ($SnCl_2 \cdot 2H_2O$) in 100 ml of concentrated hydrochloric acid. Prepare this reagent fresh every three months.

e) Lead acetate-impregnated cotton Soak cotton in a saturated solution of lead acetate, squeeze out the excess solution, and dry in a vacuum at room temperature.

f) Sample solution Transfer 1.00 gm of sample to the generating flask (Figure 1) and carefully add 5 ml of concentrated nitric acid. Add one or two small glass beads and cautiously add 2 ml of concentrated sulfuric acid in small increments. Place a stemless funnel in the neck of the flask and place the flask on an electric hot plate maintained at a moderate temperature. Regulate the heating in a manner to prevent excessive foaming, and rotate the flask occasionally to prevent the sample from caking. Maintain oxidizing conditions at all times during the digestion by adding small quantities of nitric acid whenever the mixture turns brown or darkens. Record the total volume of nitric acid used in this procedure. Continue the digestion until the organic matter is destroyed, fumes of sulfuric acid are copiously evolved, and the solution becomes colorless or retains only a light straw color. Cool the flask, cautiously add 10 ml of distilled water, and again evaporate to strong fuming, repeating if necessary to remove all of the nitric acid. Cool, dissolve the residual liquid in about 10 ml of distilled water, wash the funnel and sides of the flask with a few ml of water, and dilute to 35 ± 2 ml.

g) Procedure Add 20 ml of dilute sulfuric acid (1:5 solution) to the flask.

h) By means of suitable transfer pipets, add 2 ml of 15 per cent aqueous potassium iodide and 0.5 ml of the stannous chloride solution. Swirl gently to mix and allow the solution to stand for 30 minutes at room temperature.

i) Pack the scrubber tube of the apparatus with two plugs of the lead acetate-impregnated cotton in a manner to insure that all evolved vapors will be contacted. Lubricate the joints b and d with a suitable stopcock grease and connect the scrubber unit to the absorber tube e.

j) By means of a suitable transfer pipet, introduce 3 ml of the silver diethyldithiocarbamate solution to the absorber tube.

k) Add 3.0 gm of granular zinc (No. 30 powder) to the mixture in the generator flask and immediately insert the standard-taper joint in the flask.

l) Allow the evolution of hydrogen and the color development to proceed at room temperature (not below 25°C) for 45 minutes, or until gas evolution is complete, gently swirling to the flask at 10-minute intervals. Take care to prevent the absorber solution from being drawn back into the scrubber tube when the gas evolution is complete.

m) Disconnect the absorber tube from the generator and scrubber units and transfer the silver diethyldithiocarbamate solution to a 1-cm Beckman Model B Spectrophotometer cell.

n) Determine the absorbance of the solution at a wavelength of 525 mμ, based on a reading of zero for a blank of the original silver diethyldithiocarbamate solution. The absorbance must be less than that obtained from 3.0 ml of standard arsenic solution when treated as described below, indicating less than 3 ppm arsenic (As).

o) Standard color solution Perform the oxidation procedure described in paragraph f, using 3.0 ml of the standard arsenic solution in place of the sample. Evaporate the 3.0 ml to dryness and oxidize with 2 ml of concentrated sulfuric acid plus the total volume of nitric acid used in paragraph f. After oxidation is complete, dilute to 35 ± 2 ml with distilled water.

p) Develop and measure the color absorbance produced from this solution, following the procedure described in paragraph g through n.

q) This standard color must be prepared for each freshly-prepared standard arsenic solution, paragraph b.

4 HEAVY METALS

CAUTION! Use only glass containers free of soluble lead salts.

a) Lead stock solution Transfer 0.1598 gm of reagent grade lead nitrate, $Pb(NO_3)_2$, to a 1000-ml glass-stoppered volumetric flask containing 100 ml of distilled water and one ml of concentrated c.p. nitric acid. Swirl to effect solution, dilute to the mark with distilled water, stopper the flask, and mix thoroughly. Reserve this solution as the lead stock solution.

b) Standard lead solution Introduce 10 ml of the lead stock solution, paragraph a, into a 100-ml glass-stoppered volumetric flask by means of a suitable transfer pipet. Dilute to the mark with distilled water, stopper the flask, and mix thoroughly. This standard solution must be prepared fresh daily. A one-ml portion of this solution is equivalent to 10 ppm of lead when using a one-gm sample.

c) Procedure Transfer 1.0 gm of the sample weighed to the nearest 0.01 gm to a suitable beaker containing 25 ml of distilled water. Mix thoroughly to effect solution.

d) Add portions of dilute hydrochloric acid (1:9) until the solution is just slightly acid to litmus.
e) Transfer the solution to one of two matched 50-ml Nessler tubes. Rinse the beaker with a few ml of distilled water and add the rinsings to the tube.
f) Into the second tube pipet 1.0 ml of the standard lead solution. Add 25 ml of distilled water and make the solution slightly acid to litmus by the addition of small portions of dilute hydrochloric acid (1:9).
g) To each tube add 10 ml of a freshly prepared saturated aqueous solution of hydrogen sulfide, stopper with a clean rubber stopper, mix thoroughly, and allow to stand for 10 minutes at room temperature.
h) Compare the color of the sample with that of the blank by viewing down through the tubes against a white surface. If the color of the sample is darker than that of the standard, the heavy metals content is in excess of 10 ppm.

5 POTASSIUM COMPOUNDS

a) Sodium bitartrate solution Dissolve 1 gm of sodium bitartrate in sufficient distilled water to make a 10-ml volume. This solution must be freshly prepared.
b) Procedure Prepare a clear, saturated aqueous solution of the sample in a suitable flask.
c) Measure 5 ml of this solution into a suitable glass-stoppered graduate.
d) Add 2 or 3 drops of the sodium bitartrate solution, stopper the graduate, and mix thoroughly.
e) Examine the solution for any evidences of turbidity which indicate the presence of potassium compounds.

"NIACET" SODIUM ACETATE, ANHYDROUS

1 SODIUM ACETATE

a) Crystal violet indicator, 0.1 per cent (wt/volume) in acetic acid. Dissolve 0.1 gm of crystal violet in 100 ml of glacial acetic acid.

b) Standard 0.1 N perchloric acid in acetic acid Mix 8.5 ml of 70 per cent perchloric acid with 500 ml of glacial acetic acid and 30 ml of acetic anhydride in a 1000-ml glass-stoppered volumetric flask. Cool the solution and add glacial acetic acid to make 1000-ml and mix thoroughly. Allow the solution to stand for 1 day, for the excess acetic anhydride to be combined, and determine the water content by the Kark Fischer Method. If the water content exceeds 0.05 per cent, add more acetic anhydride, but if the solution contains no titratable water, add sufficient water to make the content between 0.02 and 0.05 per cent water. Allow to stand for one day, and again determine the water content by titration. Standardize the solution in the usual manner.

c) Procedure Transfer 0.28 to 0.32 gm of sample weighed to the nearest 0.1 mg to each of two glass-stoppered Erlenmeyer flasks containing exactly 50 ml of glacial acetic acid. Swirl to effect complete solution. The sample will dissolve more readily if the glass-stoppered flasks are held in hot water and swirled. Reserve an additional 50 ml of glacial acetic acid for a blank determination.

d) Cool the flasks and contents, add exactly 2 drops of crystal violet indicator, and titrate with the standard perchloric acid in acetic acid to the first permanent blue-green color.

e) Calculation

$$\frac{(A-B)N \times 8.204}{\text{gm sample}} = \text{sodium acetate, \% by weight}$$

A = ml of N normal perchloric acid in acetic acid required for the sample

B = ml of N normal perchloric acid in acetic acid required for the sample

2 ACETIC ACID

a) Transfer 10.0 gm of the sample weighed to the nearest 0.01 gm to a 250-ml Erlenmeyer flask containing 50 ml of distilled water.

b) Add 4 to 6 drops of 1.0 per cent alcoholic phenolphthalein indicator.

c) Titrate with standard 0.1 N sodium hydroxide to a pink end point permanent for at least 15 seconds.

d) Calculation

$$\frac{AN \times 6.005}{\text{gm sample}} = \text{acetic acid, \% by weight}$$

A = ml of N normal NaOH required

3 WATER

a) Sulfur dixodie-iodine prepared with methyl CELLOSOLVE strong reagent (water factor = 0.40). Transfer 2700 ml of methyl CELLOSOLVE, specification 1-11B1-1 or equivalent, to a 4-liter bottle having a 29/42 ground-glass neck. Add 380 gm of reagent-grade iodine, stopper the bottle securely and place on a mechanical roller for 30 minutes or until the iodine is completely in solution. Add 1200 ml of freshly distilled anhydrous pyridine. By means of a suitable glass tube extending to the bottom of the bottle add 290 gm of refrigerant-grade sulfur dioxide dried by passing thru concentrated sulfuric acid. Allow the contents of the bottle to stand for 72 hours before use. Standardize as directed in 7-1P4-8a either by visual end point or by electrometric end point.

b) Procedure Transfer 50 ml of anhydrous methanol to each of three 250-ml glass-stoppered Erlenmeyer flasks.

c) From a buret add the SO_2-I_2 reagent to each flask until a permanent light reddish brown color is obtained. Match the colors of the solutions in transmitted light by the dropwise addition of the reagent.

d) Reserve one of the flasks as a blank.

e) Into each of the other flasks introduce the following specified sample size weighed to the nearest 0.1 mg.

Grade	Sample wt, gm
NIACET Sodium Acetate, Anhydrous	2.8 to 3.0
NIACET Sodium Acetate, 60% N.F. Grade	0.1 to 0.12
NIACET Sodium Acetate, 60% Technical Grade	0.1 to 0.12
NIACET Sodium Acetate, Anhydrous, Photographic Grade	2.8 to 3.0

f) Immediately titrate with the SO_2-I_2 reagent until the light reddish brown color persists, using the blank as a guide to the proper color at the end point.

NOTE: This analysis may be performed by means of the electrometric end point using the apparatus shown in 7-1P4-8a.

g) Calculation

$$\frac{A \times F}{\text{gm sample}} = \text{water, \% by weight}$$

A = ml of SO_2-I_2 reagent required for the sample
F = factor, gm of water equivalent to 100 ml of the reagent

4 HALIDES

a) Ferric indicator Dissolve 80 gm of ferric ammonium sulfate in 1000 ml of distilled water. Carefully add 5 ml of c.p. nitric acid and mix thoroughly.

b) Procedure Transfer 20.0 gm of the sample weighed to the nearest 0.01 gm to a 400-ml beaker containing 100 ml of distilled water. Stir thoroughly to effect complete solution.

c) Add 2 ml of the ferric indicator and a sufficient amount of nitric acid to destroy the red color, leaving the solution colorless or light yellow.

d) From a buret add 1 ml of standard 0.1 N potassium thiocyanate, making the solution red again.

e) Titrate the solution with standard 0.1 N silver nitrate until the red color disappears and add 2 or 3 ml of the silver nitrate in excess. Record the total addition.

f) Add 5 ml of reagent grade nitrobenzene and stir the solution vigorously with a stirring bar and magnetic stirrer

g) Titrate the solution with standard 0.1 N potassium thiocyanate to a faint orange-red color.

h) Calculation

$$\frac{(AN-A'N') \times 5.845}{\text{gm sample}} = \text{halides, per cent by weight, as NaCl}$$

A = total ml of N normal $AgNO_3$ added and used in titration

A' = total ml of N' normal KSCN added and used in titration

5 CARBONATES

a) Barium chloride solution Add 25.5 gm of technical grade barium chloride, $BaCl_2$, to a 1000-ml volumetric flask containing 18.5 ml of 50 per cent aqueous sodium hydroxi NaOH, and 500 ml of distilled water. Dilute to the mark with additional distilled water, stopper, and mix thoroughly. Allow the solution to stand to permit settling. Use only the upper clear solution in this determination.

b) Procedure Add 50 ml of the barium chloride solution to a suitable Erlenmeyer flask.

c) Transfer 5.0 gm of the sample weighed to the nearest 0.1 gm to the flask and swirl to effect complete solution.

d) Examine the solution for any evidences of excessive turbidity which indicate the presence of carbonates.

6 IRON

a) Standard iron solution Dissolve 0.702 gm of ferrous ammonium sulfate, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, in 10 ml of 10 per cent aqueous sulfuric acid and dilute to 1000 ml with distilled water. Mix thoroughly. One ml of this solution contains 0.1 mg Fe.

b) Procedure Transfer 4.0 gm of the sample, weighed to the nearest 0.1 mg, to a suitable beaker containing 20 ml of distilled water and 15 ml of concentrated c.p. hydrochloric acid. Mix thoroughly to effect complete solution.

c) Transfer this solution to a 50-ml glass-stoppered mixing cylinder. Wash the beaker once with 5 ml of distilled water and add the washing to the mixing cylinder.

d) To a second 50-ml glass-stoppered mixing cylinder, add 25 ml of distilled water and 15 ml of concentrated c.p. hydrochloric acid. Reserve as the blank.

e) To each cylinder add one or two crystals of ammonium persulfate and 5 ml of a 10 per cent aqueous solution of potassium thiocyanate. Mix the contents of each cylinder thoroughly.

f) Transfer the contents of each cylinder to respective 23-ml cylindrical absorption cells and determine the absorbance of the sample solution on the Fisher Electrophotometer, using the 525B filter.

g) From a previously prepared calibration curve, read the ppm iron corresponding to the absorbance.

h) Calibration To respective 50-ml glass-stoppered mixing cylinders containing 20 ml of distilled water and 2 ml of concentrated c.p. hydrochloric acid, add 0.08, 0.10, 0.20, and 0.40 ml of the iron standard. Reserve a fifth cylinder as the blank. Dilute the contents of each cylinder to 40 ml with distilled water.

i) To each cylinder add one or two crystals of ammonium persulfate and 5 ml of 10 per cent aqueous potassium thiocyanate and mix the contents of each cylinder thoroughly.

j) Transfer the contents of each cylinder to respective 23-ml cylindrical absorption cells and measure the absorbance of each standard against the blank, using the Fisher Electrophotometer and the 525B filter.

k) Plot the ppm iron (based on a 4-gm sample) against the absorbance readings.

7 pH OF A 10 PER CENT SOLUTION

a) Transfer 10.0 gm of the sample weighed to the nearest 0.1 gm to a suitable beaker containing 90.0 gm of distilled water. Mix thoroughly.

b) Determine the pH of this solution at 25°C by means of a Beckman or similar pH meter.

8 APPEARANCE

a) Observe a portion of the sample in transmitted light. The sample shall be a white powder in form.

ACETIC ACID, GLACIAL - FOOD CHEMICALS CODEX GRADE

		Specification Limits	Method
1	Acetic acid	99.8 per cent by weight, minimum	1B-3A2-1
2	Acetaldehyde	0.03 per cent by weight, maximum	1B-3A2-1
3	Formic acid	0.15 per cent by weight, maximum	1B-3A2-1
4	Freezing point	16.2°C, minimum	1B-3A2-1
5	Sulfates	0.0006 per cent by weight, maximum, calculated as SO_4	1B-3A2-1
6	Chlorides	0.0004 per cent by weight, maximum, calculated as Cl	1B-3A2-1
7	Sulfurous acid	None	1B-3A2-1
8	Heavy metals	0.0010 per cent by weight (10 ppm), maximum as Pb	1B-3A2-1
9	Arsenic	3 ppm, maximum, as As	1B-3A2-62
10	Iron	0.0005 per cent by weight (5 ppm), maximum	1B-3A2-1
11	Permanganate time test	2 hours, minimum	1B-3A2-62
12	Water	0.2 per cent by weight, maximum	1B-3A2-1
13	Nonvolatile matter	0.005 per cent by weight, maximum	1B-3A2-62
14	Color	15 platinum-cobalt, maximum	1B-3A2-1
15	Suspended matter	Substantially free	1B-3A2-1

ACETIC ACID, GLACIAL - FOOD CHEMICALS CODEX GRADE

1 ARSENIC

a) Determine the arsenic content of the sample by the procedure described in 30-29C33-2, Arsenic, Determination by the Silver Diethyldithiocarbamate Colorimetric Method.

b) Use 1.00 ± 0.01 gm of sample in preparing the sample solution.

c) Report the arsenic content as ppm As.

2 PERMANGANATE TIME TEST

a) Introduce 2 ml of the sample into a suitable test tube by means of a suitable transfer pipet.

b) Add 10 ml of distilled water and 0.1 ml of 0.1 N potassium permanganate.

c) Allow the tube to stand for 2 hours at room temperature.

d) Observe the color of the solution. The pink color shall not entirely change to brown within 2 hours.

3 NONVOLATILE MATTER

a) Transfer 100 ml of the sample to a suitable weighed platinum evaporating dish.

b) Evaporate to dryness on a suitable steam bath.

c) Place the dish in an oven maintained at 105 to 110°C for 60 minutes or until constant weight is attained.

d) Cool the dish in a desiccator and weigh to the nearest 0.1 mg.

e) Calculation

$$\frac{\text{gm residue}}{\text{sp gr}} = \text{nonvolatile matter, \% by weight}$$

rlp

ACETIC ACID, GLACIAL

1 ACETIC ACID

a) Using the freezing point, as determined in Section 4, below, determine the purity by reference to the following table:

Freezing Point, °C	Acetic Acid, % by weight
16.63	100.00
16.6	99.98
16.5	99.93
16.4	99.88
16.3	99.83
16.2	99.78
16.1	99.72
16.0	99.67
15.9	99.62
15.8	99.57
15.7	99.52
15.6	99.47
15.5	99.41
15.4	99.36
15.3	99.30

b) Determine the purity to the nearest 0.03 per cent by interpolation in the table and report to the nearest 0.1 per cent, following the accepted rounding-off procedure.

c) Reference ASTM E 302-68

2 ACETALDEHYDE

a) Determine the specific gravity of the sample at room temperature by means of a suitable hydrometer calibrated to give the apparent specific gravity at t/20°C.

b) Transfer 50 ml of distilled water to each of two 250-ml glass-stoppered Erlenmeyer flasks.

c) Introduce 10 ml of approximately 1.2 per cent aqueous sodium bisulfite into each flask using a suitable transfer pipet.

d) Reserve one of the flasks for the blank determination.

e) Into the second flask pipet 20 ml of the sample. Replace the stopper and shake the flask vigorously.

f) Allow the sample and blank to stand at room temperature for 30 minutes.

g) Titrate each with standard 0.1 N iodine to the first yellowish-brown end point.

h) Calculation

$$\frac{(B - A)N \times 2.2}{20 \times \text{sp gr}} = \text{acetaldehyde, \% by weight}$$

A = ml of N normal iodine required for the sample
B = ml of N normal iodine required for the blank

FORMIC ACID

PRECAUTION! Lead tetraacetate is both poisonous and corrosive. Avoid inhalation of dust or skin contact.

a) Lead tetraacetate, 0.04 M Dissolve 100 gm of reagent-grade potassium acetate in a mixture of 50 ml of acetic anhydride and 500 ml of glacial acetic acid contained in a 1-liter beaker under a fume hood. Cool the contents to room temperature and add the solution to a 1-liter volumetric flask containing 18.0 gm of lead tetraacetate. Swirl to effect solution, adding more acetic acid if necessary. Dilute to 1-liter with acetic acid and filter through a fluted Whatman No. 12 filter paper containing a small piece of glass wool. Store the filtered reagent in a brown glass bottle.

b) Procedure Into each of four 250-ml glass-stoppered Erlenmeyer flasks pipet 10 ml of the 0.04 M lead tetraacetate. Reserve two of the flasks as blanks.

c) Into each sample flask pipet the amount of sample shown in the following table:

Expected formic acid concentration	Sample size, ml
0 to 0.05	20
0.05 to 0.15	5

Swirl the flasks and allow to react 30 minutes at room temperature.

d) Add 10 ml of 15 per cent aqueous potassium iodide and 75 ml of 20 per cent aqueous potassium bromide. Swirl to dissolve the precipitate and titrate immediately with 0.02 N sodium thiosulfate to a starch end point.

e) Calculation

$$\frac{(B - A)N \times 2.30}{\text{ml sample} \times \text{sp gr}} = \text{formic acid, \% by weight}$$

A = ml of N normal sodium thiosulfate required for the sample

B = average ml of N normal sodium thiosulfate required for the blank

4 FREEZING POINT

a) Thermometer A mercury, etched-glass thermometer having a range of 0 to 30°C, graduated in 0.1°C divisions and calibrated for 76-mm immersion. The over-all length should be approximately 368 mm. ASTM thermometer 90C is recommended.

b) Sample container A standard heat-resistant glass test tube with lip, 25 mm OD, and 150 mm in length.

c) Air jacket A standard heat-resistant glass test tube with lip, 38 mm OD and 200 mm in length. Fit with a cork or rubber stopper bored with a hole into which the sample container may be inserted up to the lip.

d) Stirrer 1-mm diameter (B and S gage 18) corrosion-resistant wire bent into a series of three loops about 25 mm apart at right angles to the shaft so that the stirrer will move freely in the space between the thermometer stem and the inner wall of the sample container. The shaft may be of any convenient length and shall pass through a 2-hole cork stopper, the center hole of which holds the thermometer. The upper end of the stirrer may be attached to a reciprocating device or formed into a loop to facilitate handling during stirring.

e) Procedure Pour the sample into the sample container to a depth of approximately 100 mm. Fit the sample container into the air jacket and immerse the assembly in a slurry of ice and water as shown in Figure 1.

f) Allow the sample to cool while stirring at a rate of about 30 strokes per minute. Discontinue stirring after an appreciable amount of crystals has formed.

g) Observe and record the temperature at regular intervals, estimating to the nearest 0.01°C until the temperature rises from minimum to a maximum, and finally begins to drop. The maximum temperature reading, after super-co[illegible]

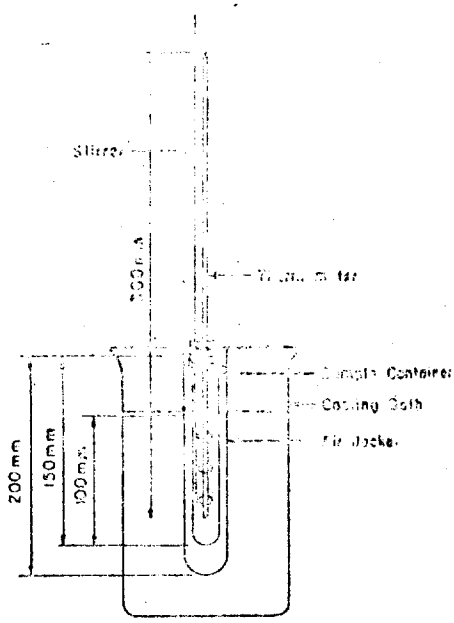

FIGURE I

APPARATUS FOR DETERMINING FREEZING POINT

NOTE! Readings should be taken 10 seconds apart in order to establish that the temperature is at the maximum level, and should continue to be taken until the first drop in temperature is noted. If the temperature rise exceeds 0.5°C, remelt the material by gently warming it in the container and repeat the determination. Seed with two or three small crystals of frozen sample when the temperature is 0.2 to 0.3°C below the expected freezing point. Crystals of the sample can be obtained by freezing a small quantity in a test tube immersed directly in the cooling bath.

h) Report as the freezing point, to the nearest 0.05°C, the average of the highest readings (at least three) made at 10-second intervals at the maximum level of temperature.

i) Reference: ASTM D 1493 and ASTM E 302

5 SULFATES

a) Standard sulfate solution Dissolve 0.0463 gm of anhydrous c.p. sodium sulfate, Na_2SO_4, in a 1000-ml glass-stoppered volumetric flask containing 100 ml of distilled water. Dilute to the mark with distilled water, stopper and mix thoroughly. A 10-ml aliquot of this solution is equivalent to 0.0006 per cent SO_4 when using a 50-ml sample.

b) Procedure Introduce 50 ml of the sample into a clean 100-ml porcelain evaporating dish.

c) Add one ml of a one per cent solution of sodium carbonate in distilled water, and evaporate the solution to dryness on a water bath maintained at 98 ± 2°C.

d) Dissolve the residue using one ml of 1 N hydrochloric acid.

e) Transfer the contents of the dish to one of two 50-ml Nessler tubes using three separate 10-ml portions of distilled water.

f) To the second tube add 30 ml of distilled water, one ml of one per cent sodium carbonate solution, one ml of 1 N hydrochloric acid, and 10.0 ml of the standard sulfate solution.

g) To each tube add two ml of a 10 per cent solution of barium chloride in distilled water, dilute to the mark with distilled water, and mix thoroughly.

h) Allow the tubes to stand for 10 minutes at room temperature and compare the contents by viewing vertically through the tubes against a dark background. If the turbidity of the sample tube is greater than that of the standard, the sulfates content is in excess of 0.0006 per cent as SO_4.

6 CHLORIDES

a) Standard chloride solution Dissolve 0.0160 gm of c.p. sodium chloride, NaCl, in 100 ml of distilled water contained in a 1000-ml glass-stoppered volumetric flask. Dilute to the mark with distilled water, stopper, and mix thoroughly. 4.3 ml of this solution is equivalent to 0.0004 per cent chlorides as Cl when using a 10-ml sample.

b) Procedure Introduce 10 ml of the sample into one of two 50-ml Nessler tubes containing 25 ml of distilled water.
c) Into the second tube introduce 4.3 ml of the standard chloride solution by means of a graduated pipet or buret.
d) To each tube add three ml of concentrated c.p. nitric acid, one ml 0.1 N silver nitrate, dilute to the mark with distilled water, and mix thoroughly.
e) Compare the contents by viewing vertically through the tubes against a dark background. If the turbidity of the sample tube is greater than that of the standard, the chlorides content is in excess of 0.0004 per cent as Cl.

7 SULFUROUS ACID
a) Transfer 300 ml of distilled water to a 500-ml Erlenmeyer flask.
b) Add 5 ml of 1.0 per cent aqueous starch indicator and add standard 0.1 N iodine dropwise to the first blue end point.
c) Introduce 50 ml of the sample by means of a graduate.
d) Using a 10-ml buret, slowly titrate with standard 0.1 N iodine to the first blue end point. If this titration does not exceed 0.1 ml, the sulfurous acid content is nil.

8 HEAVY METALS
a) Standard lead solution Dissolve 0.1680 gm of c.p. lead nitrate, $Pb(NO_3)_2$, in 50 ml of distilled water contained in a 100-ml glass-stoppered volumetric flask. Add one ml of concentrated c.p. nitric acid, dilute to the mark with distilled water, and mix thoroughly. Pipet 5 ml of this solution into a 1000-ml glass-stoppered volumetric flask, dilute to the mark with distilled water, and mix. Prepare the second solution fresh daily. 20 ml of this solution is equivalent to 0.0010 per cent heavy metals as lead when using a 10 ml sample.
b) Procedure Pipet 10 ml of the sample into a clean Pyrex or platinum evaporating dish.
c) Add one ml of a one per cent solution of sodium carbonate solution in distilled water and evaporate to dryness on a water bath maintained at 98 ± 2°C.
d) Add one ml of 1 N acetic acid to dissolve the residue and transfer the solution to one of two 50-ml Nessler tubes using three separate 5-ml portions of distilled water to effect the transfer.
e) To the second tube add 15 ml of distilled water, one ml of 1 N acetic acid and exactly 20 ml of standard lead solution.
f) To each tube add 10 ml of a saturated solution of hydrogen sulfide in distilled water, dilute each to the mark with distilled water, and mix.
g) Compare the contents by viewing vertically through the tubes against a white background. If the brown color of the sample solution is greater than that of the standard, the heavy metals content is in excess of 0.0010 per cent.

IRON

a) Standard iron solution Dissolve 0.7338 gm ferrous ammonium sulfate, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 25 ml of distilled water contained in a 100-ml volumetric flask. Cautiously add 10 ml of concentrated c.p. sulfuric acid, cool to room temperature, and dilute to the mark with distilled water. Pipet 10 ml of this solution into a 1000-ml volumetric flask, add one ml of concentrated c.p. sulfuric acid, dilute to the mark with distilled water, and mix. Five ml of this solution is equivalent to 0.0005 per cent iron as Fe when using a 10 ml sample.

b) Procedure Pipet 10 ml of the sample into a Pyrex evaporating dish containing one ml of a one per cent solution of sodium carbonate in distilled water.

c) Evaporate the contents of the dish to dryness on a hot water bath.

d) Dissolve the residue in two ml of concentrated c.p. hydrochloric acid and quantitatively transfer the solution to one of two matched 50-ml Nessler tubes using 35 ml of distilled water to effect the transfer.

e) To the second tube add 35 ml of distilled water, two ml of concentrated c.p. hydrochloric acid, and 5.0 ml of the standard iron solution.

f) To each tube add 30 to 50 mg of reagent grade ammonium persulfate crystals, three ml of a 5 per cent solution of ammonium thiocyanate in distilled water, dilute to the mark with distilled water, and mix.

g) Compare the colors of the sample and standard solutions by viewing vertically down through the tubes against a white background. If the red color of the sample is greater than that of the standard, the iron content is in excess of 0.0005 per cent as Fe.

WATER

a) Transfer 50 ml of glacial acetic acid to each of two dry 250-ml glass-stoppered Erlenmeyer flasks.

b) Titrate the contents of each flask with SO_2-I_2 reagent to the same light reddish brown color.

c) Reserve one of the flasks as a blank.

d) Into the second flask introduce 15 ml of the sample by means of a suitable transfer pipet.

e) Titrate immediately with the SO_2-I_2 reagent until the color matches that of the blank.

f) Calculation

$$\frac{A \times F}{15 \times sp\ gr} = \text{water, \% by weight}$$

A = ml of SO_2-I_2 required for the sample
F = factor, gm of water equivalent to 100 ml of reagent

g) Reference 31-29W1-1

11 COLOR

a) Transfer 100 ml of the sample to a tall-form Nessler tube.

b) Compare the color of the sample with those of a series of platinum-cobalt standards of equal volume in matched 100-ml Nessler tubes. View vertically down through the tubes against a white background.

c) Report the exact color of the sample in platinum-cobalt units.

d) Reference 5-J1-1

12 SUSPENDED MATTER

a) Invert a bottle of the sample and examine by transmitted light.

IN VITRO STUDIES ON OXIDATIVE AND LIPID METABOLISM IN THE IMMATURE MACAQUE MONKEY

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INTRODUCTION

A study of the effects of diets differing qualitatively in fatty acid composition on growth and development, on serum lipid fractions, and on cardiovascular system pathology in young macaque monkeys (*Macaca mulatta*) has been reported in a previous paper[1]. Compared with monkeys fed a diet containing vegetable oils, the animals fed an all butter-fat diet revealed higher serum cholesterol and lipid phosphorus values. Lipoprotein studies by ultracentrifugal techniques revealed a highly significant difference between the two groups of animals. Those fed butterfat had distinct elevations in the S_f 0–12, 12–20 and 20–200 fractions. Histological characteristics of the vascular systems of both groups of animals were consistent with predegenerative lesions similar to those observed in human infants and children.

In an attempt to elucidate the biochemical mechanisms responsible for these changes, *in vitro* experiments were designed that could be conducted at the time the animals were killed. This paper reports the findings on oxygen consumption, carbon dioxide production, tissue lipid levels and the incorporation of a variety of ^{14}C-labeled substrates into CO_2 and tissue lipids. The present results represent a survey of the metabolic activities of these unique animal preparations with a view to establishing patterns of metabolic activity in primate tissue and of better directing future research studies on similar animals.

It is shown that liver, adrenal and kidney cholesterol levels can be changed by diet but that oxygen utilization of liver tissue and/or carbon dioxide production is not concomitantly changed. Biosynthetic pathways were influenced by the diets, liver cholesterol labeling (biosynthesis) being inversely related to tissue cholesterol levels.

MATERIALS AND METHODS

The animals were immature Rhesus monkeys (*Macaca mulatta*) obtained from documented pregnancies at the gestational age of 168 ± 4 days and were fed from

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birth on liquid diets containing 15 % by weight synthetic formula solids. The fat of one diet was derived very largely from non-hydrogenated vegetable oils; that of the other, entirely from cow's milk. The vegetable-oil formula consisted of an almost fat-free cow's milk preparation in which a mixture of corn oil, olive oil and cocoanut oil was substituted for the butterfat; the latter represented less than 2 % of the fat in the final product. The milk-fat formula was identical except that the fat source remained unaltered. These diets were selected to cover a range of fat compositions comparable to those commonly found in the diet of infants and children on this continent. The quantity of carbohydrate, protein and fat contained in these formulae closely approximated that of human and monkey breast milk.

The diets were fed in isocaloric amounts for 350 days. The composition of the diets and the nutritional and anatomical findings are documented in the previous paper[1]. Six animals were maintained on the milk-fat diet and five on the vegetable-

TABLE I

LIPID COMPOSITION OF THE DIETS
(g/100 g dry diet)

Lipid fraction	*Diet*	
	Vegetable-fat	*Milk-fat*
Saponifiable (fatty acid)	24.4	19.1
Non-saponifiable		
By weight*	0.55	0.72
Sterol (as cholesterol) by colorimetry		
Liebermann-Burchard (*dig*)**	0.149	0.081
Liebermann-Burchard (*non-dig*)**	0.155	0.100
Zlatkis-Zak (*dig*)**	0.141	0.085

* Residue from the petroleum ether extract of the saponified diet taken to dryness, taken up in 95 % ethanol and aliquots dried for weight.
** *dig* = after precipitation with digitonin; *non-dig* = without previous precipitation with digitonin.

TABLE II

DAILY INTAKE OF FOOD AND LIPID BY ANIMALS ON THE TWO DIETS
(g/kg body weight/day)

Daily intake	*Diet**	
	Vegetable-fat	*Milk-fat*
Food (dry weight)	26.8–44.1	31.5–51.7
Fatty acid	6.6–10.8	6.0–9.9
Non-saponifiable**	0.15–0.24	0.23–0.37
Digitonin precipitable sterol, estimated colorimetrically	0.04–0.07	0.025–0.041

* Minimum and maximum values.
** Total petroleum-ether soluble, ethanol soluble, non-saponifiable material.

fat diet. As stated in the previous paper[1], the amount of food, as calories, declined from an average of 215 to 130 cals/kg/day during the year of observation.

Table I records the lipid composition of the diet as determined by our analysis and Table II the intake of the two groups of animals on the respective diets. The intake of fatty acids was similar in the two groups, but the sterol intake was somewhat higher in the milk-fat group when judged on a weight basis.

The animals were fed two hours before killing in order to favor active lipid synthesis in the subsequent *in vitro* studies[2]. Animals were killed by exsanguination while under nembutal anesthesia. It has been shown[3] that lipogenic reactions of the rat are not influenced by nembutal. At the time of death, multiple tissue samples were taken for the metabolic studies and used immediately.

TABLE III

NUMBERS OF ANIMALS USED, TRACERS FOLLOWED AND TISSUES STUDIED

Tracer	*Liver*		*Muscle*						*Adrenal*		*Kidney*	
			Heart		*Leg*		*Diaphragm*					
	*M**	*V*	*M*	*V*	*M*	*V*	*M*	*V*	*M*	*V*	*M*	*V*
[1-^{14}C]Acetate	6	5	2	3	4	4	4	4	6	5	5	4
[2-^{14}C]Mevalonate	6	4							6	5	5	4
[3-^{14}C]Acetoacetate	4	4	2	2	4	4	4	4				
[1-^{14}C]Ethanol	3	4										
[2-^{14}C]Ethanol	3	4										
[2-^{14}C]Acetate	3	3										
[1, 2-^{14}C]Ethanol	1	1										
[1, 2-^{14}C]Acetate	1	1										
[1, 2-^{14}C]Acetaldehyde	1	1										

* Milk-fat (M) diet or vegetable-fat (V) diet.

Tissues studied. Liver was used as the major tissue for study and was sliced and handled as previously described for rat liver tissue[4]. Muscle tissue was studied with muscles obtained from the heart, leg and diaphragm. The heart was sliced transversely with the Stadie-Riggs assembly and the slices handled as for liver. Leg adductor muscle fibres were carefully prepared so as to minimize cellular damage[5] and diaphragm was manually cut into thin strips. The adrenal gland and kidney tissue were also sliced with the Stadie-Riggs assembly.

Labeled tracers used. ^{14}C-Acetate was used to follow both Krebs cycle oxidations and lipid biosynthetic pathways. [2-^{14}C]Mevalonate was studied because it is a more selective tracer of sterol biosynthesis, and [3-^{14}C]acetoacetate was used to gain information on ketone body metabolism. [1- and 2-^{14}C]Ethanol were studied to compare the metabolic pathways for ethanol with those of other species. Doubly labeled acetate and ethanol were compared with doubly labeled acetaldehyde, and the three metabolites, ethanol, acetaldehyde and acetate were used to extend our other studies on ethanol metabolism to monkey tissue. Since it was not possible to use all the

tracers on all the tissues, principal attention was directed toward liver and muscle tissue.

The distribution of tissues, tracers and numbers of animals is described in Table III. Tissues were often run in duplicate or triplicate with a particular tracer metabolite. Values given are the average value for such multiple runs.

In general, amounts of tissue close to 100 mg wet weight were incubated in 3 ml Krebs phosphate buffer in 15 ml Warburg flasks at pH 7.4 for one or two hours. Flasks were gassed with O_2 prior to attachment of the manometers and all tissues of a single animal were studied simultaneously in the same Warburg bath. Labeled substrate was added from flask side arms after 30 min of equilibration. KOH on paper was used in the center well of the flasks and total CO_2 was collected after terminal acidification of the medium. Following CO_2 absorption, tissues were removed, washed and saponified and the lipids were separated as previously described[1]. Fatty acids were determined by weight, non-saponifiable material by weight, and sterols by color (as cholesterol), and the results are expressed as amounts of lipid per g of tissue wet weight. Wherever feasible, the results have been treated statistically using the t test at a confidence level of 95 %. In a number of cases, a value obtained was not included in the series being reported because it was more than two standard deviations removed from the mean.

TABLE IV

OXYGEN CONSUMPTION AND CARBON DIOXIDE PRODUCTION
(μ moles/g/h)

	Diet*	Liver	Muscle			Adrenal	Kidney
			Heart	Leg	Diaphragm		
Oxygen	M	38.5(6)**	22(2)	12.2(5)	19.2(5)	25.6(5)	59.6(5)
	V	39.9(4)	33(2)	12.0(5)	23.3(5)	23.5(5)	53.7(4)
		$P > 0.1$		$P > 0.1$	$P > 0.05$	$P > 0.1$	$P > 0.1$
Carbon dioxide	M	45.9(6)					
	V	41.5(5)					
		$P > 0.1$					
R.Q.	M	0.88					
	V	0.94					

* See footnote Table III.
** Mean value and number of animals.

RESULTS AND DISCUSSION

In the discussion to follow, little reference will be made to lipid metabolism studies of others on monkeys, for equivalent young animals have not previously been available [6–8]. In a number of other studies [9–11], deficiency diets were fed or cholesterol was added to the diets [6,8,9,12,13]. Variations in dietary fat have also received attention[6,14,15], but in general, these studies have involved more extensive dietary manipulation than those reported here.

The comparative metabolic activity of six tissues of the young monkeys is given in Table IV. Oxygen utilization is shown to range from 12 to 60 μmoles/g/h, with kidney tissue being the most active and leg muscle fibres the least active. Heart muscle slices and adrenal slices were of about the same activity while liver was the second most active. The production of CO_2 ($^{12}CO_2$) is reported only for liver tissue, the only one available in large enough amounts to permit this measurement. The calculated R.Q. value for liver tissue is also presented.

The O_2 uptake by these tissues does represent the relative order of endogenous metabolism, because these *in vitro* measurements were made on tissues carefully prepared within a short period of time after removal from the animal. The presence of only tracer amounts of organic compounds in the solutions eliminates all but the contribution of endogenous metabolism to oxygen utilization.

Statistical evaluation of the results showed that the animals on milk-fat and vegetable-fat diets had tissue oxygen utilizations that were essentially identical at the 95 % confidence level. It would thus appear that the activity of the systems involved in oxygen utilization was unchanged by the diets.

In the previous paper of this series, the development of increased levels of total plasma cholesterol, phospholipid and lipoprotein was described in some detail. Blood lipids were determined only at the time of death in the present study and are recorded in Table V. A clear-cut increase is evident in both the total and free cholesterol

TABLE V

SERUM LIPIDS AT TIME OF SACRIFICE
(mg/100 ml serum)

Diet	*Number of animals*	*Cholesterol**			*Total lipid***
		Total	*Free*	*F/T*	
Milk-fat	6	291	75	0.26	666
Vegetable-fat	5	156	31	0.20	503

* Determined as digitonin-precipitable sterol, expressed as cholesterol.
** Alcohol/ether soluble, petroleum-ether soluble lipid.

levels of the milk-fat group. The total serum lipid level of this group was also increased.

In Table VI, there are presented the fatty acid and cholesterol concentrations for the six tissues studied. It can be seen that total fatty acid values range from 250 mg/g in the case of adrenal to values 1/10 of this for diaphragm. A statistical comparison of the fatty acid values for the two diet groups was made for the three tissues (liver, adrenal, kidney) in which the greater number of samples was available. There appears to be no difference between the liver fatty acid levels of the two diet groups. The differences seen between mean levels in the case of kidney and adrenal lack statistical significance, perhaps because of the small number of samples available for study.

The liver and kidney of the milk-fat animals showed a significant increase in the amounts of the cholesterol fractions present but the adrenal glands of the vegetable-fat animals had cholesterol levels significantly in excess of the milk-fat group. The liver tissue from the milk-fat animals showed a cholesterol value of 4.4 mg/g, almost twice that of the vegetable-fat animals.

Considering the differences in and the specialized functions of liver, adrenal and kidney, it is not surprising to find these large differences in cholesterol levels. In view of the well-known relationship of adrenal cholesterol and adrenal steroid hormones, some significance may be attached to the differences seen in adrenal cholesterol values on the two diets.

TABLE VI

FATTY ACID AND CHOLESTEROL LEVELS IN TISSUES (mg/g)

	Diet	Liver	Muscle			Adrenal	Kidney
			Heart	Leg	Diaphragm		
Fatty acids	M	38.9(5)*	51(2)	25(2)	24(1)	249.8(5)	95.8(4)
	V	30.9(4)	21(2)	9.6(2)	18(1)	189.0(3)	70.1(3)
P**		>0.1				>0.1	>0.1
Cholesterol	M	4.4(6)	0.96(2)	0.55(5)	0.61(5)	20.6(5)	2.8(5)
	V	2.4(3)		0.31(2)	0.76(2)	75.9(2)	2.1(2)
P**		<0.05		>0.05	>0.1	<0.005	<0.05

* Mean value and number of samples.
** A P value of <0.05 indicates a significant difference between mean values at the 95% confidence level.

TABLE VII

% INCORPORATION INTO LIVER FRACTIONS (Incorporation × 100/dose/h/g tissue)

Tracers	Fatty acid		Cholesterol		CO_2	
	M	V	M	V	M	V
[1-14C]Acetate	0.124(6)*	0.176(3)	0.049(6)	0.237(3)	5.40(6)	5.54(4)
	$P > 0.1$		$P < 0.05$		$P > 0.1$	
[2-14C]Mevalonate	0.506(6)	0.284(5)	4.86(6)	6.49(5)	0.26(6)	0.77(5)
	$P > 0.1$		$P < 0.025$		$P < 0.025$	
[3-14C]Acetoacetate	0.078(4)	0.058(4)	0.027(5)	0.037(4)	1.71(4)	1.09(4)
	$P > 0.1$		$P > 0.1$		$P < 0.025$	
[1-14C]Ethanol	0.227(3)	1.27(2)	0.024(3)	0.945(2)	9.42(3)	7.32(1)
[2-14C]Ethanol	0.292(3)	1.07(2)	0.034(3)	0.897(2)	2.42(3)	1.91(2)
[2-14C]Acetate	0.099(2)	0.179(1)	0.028(2)	0.343(1)	2.02(2)	1.58(1)
[1, 2-14C]Ethanol	0.240(1)	0.284(1)	0.040(1)	0.330(1)		
[1, 2-14C]Acetate	0.117(1)	0.193(1)	0.019(1)	0.177(1)		
[1, 2-14C]Acetaldehyde	0.094(1)	0.115(1)	0.014(1)	0.157(1)		

* Mean value and number of animals.

Evaluation of the metabolic activity of the tissues was made with a variety of ^{14}C-labeled compounds. Major attention was given to the use of acetate, acetoacetate and mevalonate. Table VII presents data obtained with liver tissue and Table VIII similar data from muscle, adrenal and kidney tissue.

TABLE VIII

INCORPORATION OF TRACERS INTO MUSCLE, ADRENAL AND KIDNEY

	Fatty acid		*Cholesterol**		CO_2	
	*M***	*V*	*M*	*V*	*M*	*V*
Heart muscle						
[1-^{14}C]Acetate	0.032(2)	0.081(2)	0.021(2)	0.013(2)	19.6(2)	28.1(2)
[3-^{14}C]Acetoacetate	0.036(2)	0.051(2)	0.016(2)	0.015(2)	6.55(2)	16.0(2)
Leg muscle						
[1-^{14}C]Acetate	0.058(4)	0.029(4)	0.033(4)	0.025(4)	17.76(5)	19.2(4)
P	> 0.1		> 0.1		> 0.1	
[3-^{14}C]Acetoacetate	0.058(5)	0.023(4)	0.044(5)	0.018(4)	8.55(5)	7.56(4)
P	> 0.1		> 0.1		> 0.1	
Diaphragm						
[1-^{14}C]Acetate	0.060(4)	0.063(3)	0.028(5)	0.023(4)	17.0(5)	18.6(4)
P	> 0.1		> 0.1		> 0.1	
[3-^{14}C]Acetoacetate	0.30(3)	0.082(4)	0.038(5)	0.017(4)	7.16(5)	9.91(4)
P	> 0.1		> 0.1		> 0.1	
Adrenal						
[1-^{14}C]Acetate	0.133(6)	0.065(5)	0.452(5)	0.194(6)	8.1(6)	9.93(5)
P	> 0.05		> 0.05		> 0.1	
[2-^{14}C]Mevalonate	0.295(6)	0.067(5)	0.12(5)	0.064(5)	0.80(6)	0.53(5)
P	> 0.1		< 0.05		> 0.1	
Kidney						
[1-^{14}C]Acetate	0.252(5)	0.145(4)	0.06(4)	0.048(4)	27.7(5)	29.2(4)
P	> 0.1		> 0.1		> 0.1	
[2-^{14}C]Mevalonate	0.786(5)	0.80(4)	2.85(5)	2.81(4)	1.94(5)	1.44(4)
P	> 0.1		> 0.1		> 0.1	

* Non-saponifiable fraction determined colorimetrically as cholesterol.
** See footnote Table III.

The magnitude of incorporation of a precursor tracer molecule is generally taken to represent the biosynthetic activity of the preparation. Without knowledge of the size and turnover of the intermediate pools on the biosynthetic pathways, it is hazardous to compare groups of animals in relation to the magnitude of labeling. A compromise approach to the problem is to compare several metabolic systems within one group of animals to the systems in another group. This technique has been used in the past in this laboratory[16] and proved to be of considerable value.

Considering first the incorporation of labeled material into liver fatty acids: the differences between mean values of the milk-fat and vegetable-fat groups in the acetate, acetoacetate or mevalonate tested tissues were not significant. The liver tissue samples in the milk-fat group showed a lower incorporation of both the ^{14}C-ethanol compounds, but the number of tissues used was small and statistical analysis was not carried out. The data suggest that the *amounts* of fatty acid labeled (syn-

thesized) in the liver were not influenced by the fatty acid composition in the diet (the oleic acid to linoleic acid ratio was 0.75 for the vegetable-fat, 9.2 for the milk-fat diet). It has been demonstrated several times [17-19] that the feeding of unsaturated fat will lead to an increase in the synthesis of sterol, but similar studies on fatty acids are lacking.

In the previous paper[1] there were reported increased serum cholesterol levels in the milk-fat group and well-established predegenerative vascular lesions in both groups. The data of Tables V and VI show increases in both serum cholesterol and total serum lipids. Because of these findings, the data presented for cholesterol labeling become of considerable importance. Considering first the incorporation of ^{14}C into liver cholesterol, it is seen that the milk-fat group shows a lower labeling of liver cholesterol from both [1-^{14}C]acetate and from [2-^{14}C]mevalonate. The greater depression of the acetate labeling compared with the mevalonate labeling, *i.e.*, 80 %, *vs.* 25 %, is not easily explained. It is unlikely that this is due simply to the dilution of tracer caused by the presence of an increased acetate (acetyl-CoA) pool, for neither the fatty acids nor the CO_2 which arise from these precursors showed a similar depression. It is more likely that the depression is the result of homeostatic mechanisms inhibiting sterol synthesis at several stages in the presence of an increased amount of liver cholesterol (Table VI). It is also possible that the increased liver sterol is more or less directly related to the observed elevation of serum cholesterol in the milk-fat diet animals. If similar differences are proven to exist in future studies, evidence of the presence of at least two molecular sites of homeostatic control will be afforded.

It is to be further noted that except for [3-^{14}C]acetoacetate, all the other tracers showed a diminution in cholesterol labeling in the milk-fat group. [3-^{14}C]Acetoacetate occupies a unique position as a tracer of these systems in liver tissue. The activation of this keto-acid is generally stated not to take place in liver. The data of Table VII show clearly, however, that small amounts of acetoacetate are incorporated into the three fractions studied. Only in the case of $^{14}CO_2$ production there was a significant effect of diet upon acetoacetate labeling; here, in contrast to the other substances used, the milk-fat diet animals showed an increased labeling of CO_2 from acetoacetate.

From a consideration of these findings it appears necessary to conclude that the hypercholesterolemia of the milk-fat group cannot be explained as the basis of an increase in cholesterol biosynthesis, at least in the period immediately preceding the death of the animals.

It is of interest in regard to the changes seen for liver to consider the findings for the other tissues as reported in Table VIII. There appears to be no significance in the differences seen between mean values for the labeling of the fatty acids or the CO_2 fraction for any of the five tissues. The three muscle tissues activated and used acetate and acetoacetate to about the same extent for fatty acid labeling but the magnitude of these reactions was not great. The amount of acetate or acetoacetate diverted to CO_2 formation was, however, proportionately much greater in muscle than in liver, the CO_2/fatty acid labeling ratio for liver being about 45 while for

muscle it is about 400. This diversion of metabolite to oxidative pathways in muscle is in keeping with the physiological activity of the tissue. In muscle, cholesterol labeling from the C_2 or C_4 tracer was of about the same order of magnitude as the fatty acid labeling, but again, this was of a low order of magnitude. The three muscle tissues are capable of both fatty acid and cholesterol synthesis. This function appears to be a minor one on a unit weight basis, but when the size of the total muscle mass is considered, these lipid pathways account for an appreciable flow of carbon to biosynthetic reactions.

Adrenal tissue differed from muscle in its pattern of lipid labeling, for both fatty acid and cholesterol fractions contained appreciable amounts of label; moreover the diets did appear to influence the labeling here. The milk-fat animals showed more incorporation of both acetate and mevalonate into fatty acids and cholesterol than did the vegetable-fat animals, the acetate incorporation differences being not quite significant at the 95 % confidence level. The greater incorporation of mevalonate into cholesterol was significant.

Kidney slices showed a pattern of acetate utilization quite different from the other tissues. The incorporation of acetate label into CO_2 by kidney was as great as it was in heart muscle. No differences existed between the diet groups in regard to [1-^{14}C] acetate utilization. [2-^{14}C]Mevalonate use by kidney tissue was also unusual, for this tissue showed a greater $^{14}CO_2$ activity than that reported for liver (Table VII), the amounts being about one half of the activity found in the cholesterol fraction. The amount of $^{14}CO_2$ liberated from the C_2 position of mevalonate during cholesterol biosynthesis by the conventional pathway is 1/5–1/6 of the amount of the label going into the sterol fraction[20]. We have previously demonstrated[21] that kidney tissue has active cholesterologenic processes and that, at least *in vivo*, kidney tissue can take up and utilize mevalonate tracer at a rapid rate.

The adrenal slices incubated with mevalonate showed an even greater anomaly in regard to the $^{14}CO_2$ activity, for it was 8–10 times higher than the sterol fraction activity; indeed this ratio was more like that found for acetate incorporation into these two fractions. It was not practical, in these studies, to fractionate the non-saponifiable material into its various constituents. Because of this, it is likely that the radioactivity observed in the non-saponifiable fraction was contained in adrenal corticoids as well as in squalene, cholesterol, etc. The adrenal corticoids are known to arise both from acetate and from cholesterol[22,23], but the exact contribution of each of these precursor pathways is not known. When cholesterol is biosynthetized from 6 moles of [2-^{14}C]mevalonate, the label appears in positions 1, 7, 15, 22 and 27 of cholesterol and one $^{14}CO_2$ is formed. If cholesterol so labeled was in turn converted to corticoids, the ^{14}C in positions 22 and 27 would be lost and would probably appear as $^{14}CO_2$. Thus, corticoid formed from 6 moles of [2-^{14}C]mevalonate through cholesterol as an intermediate would retain only three of the ^{14}C carbons. These reactions might well then lead to CO_2 and cholesterol labeling of about equal magnitude. Any lack of conversion of the labeled cholesterol to corticoids would lead to a lesser labeling of the CO_2 fraction. The disproportionate amounts of [2-^{14}C]mevalonate that

appeared in the CO_2 fraction may also be due to the presence of an alternative pathway for mevalonate metabolism in adrenal tissue.

CONCLUSIONS

It is clear from the previous paper[1] that both groups of animals had involvement of the vascular system. It is also apparent from the present and previous data that both cholesterol levels and total lipid levels of the serum of the milk-fat animals were elevated over those of the vegetable-fat animals. Liver cholesterol levels were also elevated in the milk-fat group. Liver cholesterol labeling was, however, depressed, a point which suggests that liver cholesterologenesis was also depressed. The hypercholesterolemia is not, therefore, due to increased cholesterol synthesis and its origin remains obscure.

The two groups of animals did not have appreciably different intakes of sterol, although qualitative differences in the dietary sterol did exist. The amounts of sterol were not excessive, for it is estimated that the infant on breast feeding takes in some 0.05–0.10 g/day[24], an amount similar to the values reported in Table II. Sterol intake *per se* does not, therefore, appear to be an obvious cause of the changes seen.

The lower serum cholesterol level of the vegetable-fat animals might be due to the combined effects of a low animal-sterol intake and an effect of the plant sterols on cholesterol levels, although the low level of intake of the latter would be unlikely to give rise to pharmacological effects. The other dietary difference between these groups was in the amounts of linoleic acid present (32 % of total fatty acids in the vegetable-fat, 3 % in the milk-fat diet). The high concentration of linoleic acid in the vegetable-fat diet may explain the lower serum cholesterol levels. The same animals also had lower serum lipid phosphorus and β-lipoproteir levels[1].

At least one other aspect of sterol metabolism must be considered, namely cholesterol catabolism or turnover. We have previously shown[25] that in the intact diabetic rat[25] hypercholesterolemia might be due to a decrease in the turnover of liver cholesterol. Data on cholesterol catabolism are not available in the present studies.

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SUMMARY

The effects of diets differing qualitatively in fatty acid composition on blood and tissue lipids and upon oxidative and lipid biosynthetic pathways in monkeys (*Macaca*

mulatta) have been followed. The milk-fat diet, as compared with the vegetable-fat diet, led to higher serum cholesterol, serum total lipid and liver cholesterol values. Adrenal cholesterol was lower in the milk-fat group, but kidney cholesterol values were higher. [1-^{14}C]Acetate and [2-^{14}C]mevalonate incorporation into cholesterol in the liver was lower in the milk-fat group, so that the hypercholesterolemia observed in the latter group is not attributable to increased cholesterol synthesis. Other metabolic changes are described for the two groups.

RÉSUMÉ

Les effets de régimes différant qualitativement quant à leur composition en acides gras sur les lipides du sang et du tissu et sur le mécanisme de l'oxydation et de la biosynthèse lipidique ont été suivis chez le singe (*Macaca mulatta*). Le régime aux graisses de lait, comparé avec celui aux graisses végétales donnait lieu à des taux plus élevées de cholestérol sérique, de lipides sériques totaux et de cholestérol hépatique. Dans le groupe maintenu au régime aux graisses de la synthèse de lait, le taux de cholestérol surrénal était plus élevé tandis que le taux de cholestérol rénal était plus bas.

L'incorporation dans le foie de [1-^{14}C]acétate et de [2-^{14}C] mévalonate dans le cholestérol était moindre au sein du groupe soumis au régime aux graisses de lait, si bien à ne pas attribuer la hypercholestérolémie observée dans ce dernier groupe à une montée du cholestérol. D'autres perturbations du métabolisme ont été décrites pour les deux groupes.

ZUSAMMENFASSUNG

Es wurde untersucht, wie sich Fütterungen von qualitativ unterschiedlichem Fettsäuregehalt auf Blut- und Gewebelipide sowie auf die biosynthetischen Vorgänge im Bereich des Sauerstoff- und Lipid-Stoffwechsels beim Affen (*Macaca mulatta*) auswirkten. Verglichen mit der Pflanzenfettdiät zeigte die Milchfettdiät höhere Werte für Serumcholesterin, Totalgehalt an Lipiden im Serum, Lebercholesterin und Nierencholesterin. Dagegen waren die Suprarenalcholesterinwerte niedriger. Der [1-^{14}C]-Acetat und [1-^{14}C]-Mevalonat-Einbau im Cholesterin der Leber war in der Milchfettgruppe geringer, sodass die hier angetroffene Hypercholesterinämie nicht einer gesteigerten Cholesterinsynthese zuzuschreiben ist. Es werden noch andere Veränderungen im Stoffwechsel beider Gruppen beschrieben.

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Antigoitrogenic Effect of Sodium Acetate in Relation to P.A.B. and Sulfanilamide in Rats

by Le Van Hung and Paul Delost

Since the fundamental work of Mackenzies and Astwood, the antigoitrogenic effects of p-aminobenzoic acid and sulfanilamides have been known. Several authors have studied the histological changes produced by these substances in the thyroid; however, the mechanism of their effect is still being discussed. It seemed interesting to us to undertake two series of experiments, the principal results of which we report here:

1. We compared the effect of p-aminobenzoic acid and its derivative, acetyl, on the thyroid of adult male rats, Comentry stock. These rats were of weights around 200 g, having been subjected to different diets (I, II and III; see the table). In order to avoid its alteration, acetylated P.A.B. was mixed each day in the diet in the proportion of 2.50 g per 100 g of normal diet. The animals were sacrificed after a month on the diet; their thyroids were fixed in Zenker formol-acetic acid, then studied histologically.

a. At autopsy, the thyroids of the animals subjected to diet II containing P.A.B. were very hypertrophied in relation to the thyroids of the animals subjected to diet III containing acetylated P.A.B., the latter were identical to those of the rats subjected to the normal diet (diet I).

b. Histology showed clearly the goitrogenic effect of P.A.B. (diet II); the vesicles were fairly filled with colloid discharge, some were collapsed; the cells were hypertrophied, the number of epithelium cells was very high,

Régime (1)	I	II	III	IV	V	VI	VII	VIII
P. A. B. (2)	0	2	0	2	2	0	0	0
Acétyl P. A. B. (3)	0	0	2,5	0	0	0	0	0
Sulfanilamide (4)	0	0	0	0	0	2	2	2
Acétate Na (5)	0	0	0	12,5	24	0	12,5	24
Saccharose (6)	60	60	60	47,5	36	60	47,5	36
Saindoux (7)	13	13	13	13	13	13	13	13
Caséine (8)	20	20	20	20	20	20	20	20
Mélange salin (9)	5	5	5	5	5	5	5	5
Sciure de bois (10)	2	2	2	2	2	2	2	2

(11) Vitamines hydrosolubles et liposolubles en quantité suffisante pour chaque régime.

(12) Composition des régimes (p. 100).

1 -- Diet
2 -- P.A.B.
3 -- Acetylated P.A.B.
4 -- Sulfanilamide
5 -- Sodium acetate
6 -- Saccharose
7 -- Lard
8 -- Casein
9 -- Saline mixture
10 -- Sawdust
11 -- Hydrosoluble and liposoluble vitamins in quantities sufficient for each diet
12 -- Composition of the diets (p. 100)

the nuclei were pale. By contrast, the thyroids of the rats subjected to acetylated P.A.B. (diet III) had the histological appearance in all respects comparable to that of the thyroids in periods of activity of normal rats (diet I). Acetylated P.A.B. caused no histological alterations in the sense of hypertrophy; it possesses no goitrogenic effect on the thyroid of the rat, in the doses employed in the diet.

2. We studied the effect of sodium acetate on the thyroid of adult male rats of the same stock and weight, subjected to diets containing one of the P.A.B. goitrogenic aromatic amines or sulfanilamide. Toxic in high doses, sodium acetate may be used by the animal organism in low doses; the maximum amount of acetate tolerated by the rat is 20% to 25% in the diet. We employed doses of 12.5% and 24%, the duration of the diets being one month.

a. Sodium acetate exerts a very clear protection in relation to P.A.B.; this protection is more marked the greater, the dose of acetate. Actually,

the rats subjected to diet IV containing P.A.B. and 12.5% sodium acetate had thyroids whose histological appearances were close to that of normal thyroids in periods of activity; the epithelium is of average height, the colloid is abundant with the resorption vacuoles. If the dose of sodium acetate is 24% for the same amount of P.A.B. (diet V), the thyroid then presents a completely characteristic histological picture: very flat epithelium, nuclei small and very basophilic, homogenous colloid.

b. Sodium acetate exerts no protection in relation to sulfanilamide, even in maximum doses. The thyroids of rats subjected to diets containing only sulfanilamide (diet VI), or with 12.5% sodium acetate (diet VII), or 24% sodium acetate (diet VIII), have absolutely similar histological structures; that of very marked parenchymatous goiter, colloid discharge is total, the vesicles are completely collapsed, the cells are hypertrophied, the epithelium is very high, wrinkled in places, the nuclei are clear and turgid, the interstitial tissue is abundant. This thyroid hypertrophy is more significant than that which we obtained with P.A.B.

Discussion

The absence of thyroid alterations with acetylated P.A.B. and the protective effect of sodium acetate towards P.A.B. suggests that it is the amine function present in the molecule of the latter which is responsible for its goitrogenic effect. Probably, the absence of any protective effect of sodium acetate towards sulfanilamide must be attributed to the presence of sulfated nuclei which possess their own goitrogenic effect much more significant than the amine function. P.A.B. possessing only an amine function would be less active than sulfanilamide which besides the two amine functions possesses sulfated nuclei. Thiourea would be still more active

because along with the two amine functions, it has a CS relationship much more toxic than the sulphone nuclei of sulfanilamide. The goitrogenic effect of the sulfated nuclei and CS would be due to an inhibitive effect on the enzymatic system responsible for the tyrosin-thyroxine conversion, while that of the amine function appears to be due to the deprivation of the enzymatic system of its reactive products.

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Action antigoitrigène de l'acétate de soude vis-à-vis du P.A.B. et du sulfanilamide chez le rat,

par Le Van Hung et Paul Delost.

Depuis les travaux fondamentaux des Mackenzies et d'Astwood, on connaît l'action goitrigène de l'acide para-aminobenzoïque et des sulfamidés. De nombreux auteurs ont étudié les modifications histologiques produites par ces substances au niveau de la thyroïde ; cependant leur mécanisme d'action est encore discuté. Il nous a paru intéressant d'entreprendre deux séries d'expériences dont nous rapportons ici les principaux résultats.

(5) L. E. Casida et F. F. Mc Kenzie. *Research Bull.*, 1932, t. *170*, p. 4.

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1°) Nous avons comparé l'action de l'acide para-aminobenzoïque et de son dérivé acétylé sur la thyroïde de rats mâles adultes de race Coenentry. Ces rats d'un poids voisin de 200 g, ont été soumis à des régimes différents (I, II et III ; voir tableau). Pour éviter son altération, l'acétyl P.A.B. a été mélangé chaque jour au régime dans la proportion de 2,50 g pour 100 g de régime normal. Les animaux ont été sacrifiés après un mois de régime ; leurs thyroïdes fixées au Zenker-formol-acide acétique, puis étudiées histologiquement.

a) *à l'autopsie*, les thyroïdes des animaux soumis au régime II contenant le P.A.B, sont très hypertrophiées par rapport aux thyroïdes des animaux soumis au régime III contenant l'acétyl P.A.B. : ces dernières sont identiques à celles des rats soumis au régime normal (régime I).

Régime	I	II	III	IV	V	VI	VII	VIII
P. A. B.	0	2	0	2	2	0	0	0
[illegible] P. A. B.	0	0	2,5	0	0	0	0	0
[illegible]lamide	0	0	0	0	0	2	2	2
[illegible] Na	0	0	0	12,5	24	0	12,5	24
[illegible] rose	60	60	60	47,5	36	60	47,5	36
[illegible]ax	13	13	13	13	13	13	13	13
[illegible]	20	20	20	20	20	20	20	20
[illegible] sain	5	5	5	5	5	5	5	5
[illegible] de bois	2	2	2	2	2	2	2	2

[illegible] hydrosolubles et liposolubles en quantité suffisante pour [illegible] régime.

Composition des régimes (p. 100).

b) *L'histologie* montre bien l'action goitrigène du P.A.B. (régime II) : [illegible] vésicules sont en voie de décharge colloïde, certaines sont en collapsus ; les cellules sont hypertrophiées, l'épithélium est très élevé, [illegible] sont pâles. Par contre, les thyroïdes des rats soumis à [illegible] P.A.B. (régime III) ont un aspect histologique en tout point [illegible] à celui des thyroïdes en période d'activité des rats normaux (régime I). L'acétyl P.A.B. ne provoque pas de modifications histologiques dans le sens de l'hypertrophie ; il ne possède aucun effet [illegible] sur la thyroïde du rat, aux doses employées dans le régime.

[illegible] l'action de l'acétate de soude sur la thyroïde [illegible] de même race et de même poids, soumis à des [illegible] des amines [illegible] goitrigènes P.A.B., ou [illegible] de soude peut être utilisé [illegible] le maximum d'acétate to[illegible] de 20 à 25 p. 100 dans le régime. Nous l'avons em[illegible] durée des régimes a [illegible]

a) l'acétate de soude exerce une protection très nette vis-à-vis du P.A.B. ; cette protection est d'autant plus marquée que la dose d'acétate est plus forte. En effet, les rats soumis au régime IV contenant le P.A.B. et 12,5 p. 100 d'acétate de soude, ont une thyroïde dont l'aspect histologique est voisin de celui d'une thyroïde normale en période d'activité ; l'épithélium est de hauteur moyenne, la colloïde est abondante avec des vacuoles de résorption. Si la dose d'acétate de soude est de 24 p. 100 pour la même dose de P.A.B. (régime V), la thyroïde présente alors un repos histologique complet caractéristique : épithélium très aplati, noyaux petits et très basophiles, colloïde homogène.

b) l'acétate de soude n'exerce aucune protection vis-à-vis du sulfanilamide, même aux doses maxima. Les thyroïdes des rats soumis aux régimes contenant du sulfanilamide seul (régime VI, ou associé soit à 12,5 p. 100 d'acétate de soude (régime VII), soit à 24 p. 100 d'acétate de soude (régime VIII), ont une structure histologique absolument semblable : celle d'un goitre parenchymateux très marqué : la décharge colloïde est complète, les vésicules sont en collapsus total, les cellules sont hypertrophiées, l'épithélium est très élevé, plissé par endroits, les noyaux sont clairs et turgescents, le tissu interstitiel est abondant. Cette hypertrophie thyroïdienne est plus importante que celle que nous avons réalisée à l'aide du P.A.B.

Discussion. — L'absence de modifications histologiques thyroïdiennes avec l'acétyl P.A.B., et l'action protectrice de l'acétate de soude vis-à-vis de l'acide para-aminobenzoïque, suggèrent que c'est la fonction amine présente dans la molécule de ce dernier, qui est responsable de son action goitrigène. Il faut probablement attribuer l'absence de protection de l'acétate de soude vis-à-vis du sulfanilamide, à la présence du noyau soufré qui possède une action goitrigène propre beaucoup plus importante que la fonction amine. Le P.A.B. ne possédant qu'une fonction amine serait moins actif que le sulfanilamide qui en plus de 2 fonctions amines possède un noyau soufré. La thiourée serait encore plus active, car à côté de 2 fonctions amines elle a une liaison CS beaucoup plus toxique que le noyau sulfone du sulfanilamide. L'action goitrigène des noyaux soufré et CS serait due à un effet inhibiteur sur le système enzymatique responsable de la conversion tyrosine-thyroxine, alors que celle de la fonction amine paraît être due à la privation du système enzymatique de ses produits réactifs.

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ACETYLATION OF P-AMINOBENZOIC ACID IN THE RAT WITH A FATTY LIVER

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Various hypotheses have been put forth on the origin of the acetyl radical in an organism which are based on a whole series of biochemical research studies done in vitro. Besides pyruvate, the largest source would be higher fatty acids which when oxidized give up chains of two carbon atoms generating acetyl; a series of works recently done with C_{14} supports this hypothesis.

Having undertaken research on the acetylation of p-aminobenzoic acid in vivo in the rat, we showed that the amount of acetylation in the urine measured under certain defined condition (Le Van Hung, 153), enabled a measurement to be made of the acetylating activity of the organism.

Furthermore, the study of fatty livers brought about by a protein-free diet showed that steatosis partially restores the non-oxidation of fatty acids (see M. Levy, 1949). We undertook the following type of experiments: adult Wistar rats received one of the four diets below:

Diet I: normal containing 20% casein, 13% sawdust, 60% saccharose.

Diet II: rich in casein, 80% casein, 13% sawdust and no saccharose.

Diet III: Lipid enriched, containing 20% casein, 60% sawdust and 10% saccharose.

Diet IV: without casein and containing only 13% sawdust and 80% saccharose.

All the diets further contain a 5% McCallum saline mixture and 2% roughage. Each rat receives daily necessary quantities of vitamins (according to Griffith).

The casein-rich diet (II) possesses an energy value of 1400 calories per 100 g of the diet. Diet III seems, a priori to contain the same proportion of casein as diet I because 100 g of the normal diet have an energy value of 437 calories while 100 g of diet III contain 660, although the animals ingest quanitities of casein which are between 457 and 305 g for the same quantity of calories.

A. Experiments and Results Obtained. Rats weighing between 170 and 200 g are subjected to diets lasting 28 days, with 7 animals on each diet. The index of p-aminobenzoic acid acetylation is determined in the 28 rats before the diet, then at the 7th, 14th, 21st and 28th days following ingestion. The animals are sacrificed immediately at the end of the diet and the livers are removed. An aliquot is ground up and used to determine the oxidation index of octanoic acid by the method of Lehninger and Kennedy, and the remainder is used for the determination of the total amount of ipids.

Examination of table 1 shows:

1-That the casein-rich diet brings about no change in the degree of acetylation, nor in the index of oxidation of octanoic acid. Furthermore, this diet gave only a slight increase in the amount of total lipids (4,14%).

2-On the other hand, diets III and IV brought about great disturbances. The liver of the animals having received the diet rich in fat and poor in casein (III)(33% less than the normal diet by 100 calories) is fatty (containing about 5 times more total lipids than the normal liver). The acetylation index of these animals is lowered from the 7th day by 21%; this fall subsequently increases, but more slowly-- it is 38% at the 28th day. At this time, the index of oxidation of the octanoic acid is less than 57% of the normal index, less than 40% if the adjusted indexes are taken into consideration. Thus, steatosis is accompanied by a significant fall and of the same degree of the capacity to acetylate p-aminobenzoic acid and to oxidize octanoic acid.

Concerning diet IV, lacking in casein, similar facts are observed, but there are also some particularities. The excess of fat is significant (200 p. 100) but not as great as with diet III; nevertheless, the two enzymatic systems are more affected here: the acetylation index is lowered by 34% the seventh day, 46% at the 21st day and to a low of 72% at the 28th day: acetylation is no more than 28% of its normal value, just as the index of oxidation of octanoic acid is lower by 62% for the liver and by 57% for the corrected index of the defatted liver. Here again, the similarity (parallelism) between the two phenomena is evident.

TABLE I

Influence of the amount of protein in the diet on the degree of p-aminobenzoic acid acetylation.

1 Régime	2 Caséine contenue dans 100 calories	3 Aspect du foie	4 Teneur en lipides tot. (p. 100)	Indice d'acétylation 5					6 Indice d'oxydation de l'acide octanoïque par g non délipidé	7 Indice d'oxydation de l'acide octanoïque par g délipidé
				Au dép.	7e j.	14e j.	21e j.	28e j.		
8 1. Normal	4,57	Normal	6,7	63 ± 3	61 ± 3	59 ± 4	61 ± 2	60 ± 3	2,8 ± 0,2	3,0
9 2. Riche en caséine	14	Normal	7,7	60 ± 3	57 ± 4	56 ± 4	55 ± 3	57 ± 4	2,80 ± 0,24	3,0
10 3. Riche en graisse	3,05	Très gras 12	32	60 ± 4	47 ± 4	42 ± 3	36 ± 3	37 ± 4	1,19 ± 0,30	1,8
11 4. Sans caséine	0	Très gras 12	20	61 ± 3	40 ± 3	33 ± 4	33 ± 3	17 ± 3	10,05 ± 0,15	1,3

1-Diet
2-Casein contained in 100 calories
3-Appearance of the liver
4-Amount of total lipids (in percent)
5-Acetylation index
6-Index of oxidation of octanoic acid per fatty g
7-Index of oxidation of octanoic acid per nonfatty g
8-Normal
9-Rich in casein
10-Rich in fat
11-Without casein
12-Very fatty

TABLE II

Degree of p-aminobenzoic acid acetylation amount of total lipids and oxidation index of octanoic acid of the hepatic tissue in rats first subjected to diets II, III, IV, then put on a normal or corrective diet.

1 Nº de régime	2 14e jour après le régime déséquilibré			3 7e jour après le même régime + choline			4 14e jour après le régime normal		
	II	III	IV	II	III	IV	II	III	IV
5 Taux d'acétylation		39 ± 2	32 ± 3		48 ± 2	47 ± 3	57	46 ± 3	42 ± 2
6 Indice d'oxydation de l'acide octanoïque	2,86 ± 0,24	1,19 ± 0,31	1,05 ± 0,15		2,35 ± 0,25	2,24 ± 0,20	2,80	2,55 ± 0,17	3,00 ± 0,10
7 Teneur hépatique en lipides totaux (sur le tissu frais)	7,7	32	20		8,4	17	8,10	17	7

1-Diet number
2-14th day after the unbalanced diet
3-7th day after the same diet and choline
4-14th day after the normal diet
5-Degree of acetylation
6-Oxidation index of octanoic acid
7-Total amount of hepatic lipids (in fresh tissue)

B. It proceeds from these experiments that the oxidation of fatty acids presents itself as the great source of the radicals used in the acetylation of p-aminobenzoic acid. Diet II rich in protein and bringing about no modification either in acetylation or oxidation of octanoic acid gives formal proof that the proteins are not a source of the acetyl radicals, theory that has been held up until now. By contrast, the ingestion of a diet especially rich in glycerides in relation to proteins brings about a significant fall in the acetylating activity of the liver.

We have done experiments showing that if the animals show this type of fatty liver and are given an excess of pantothenic acid, there is no improvement in the acetylation index. Thus the fall is not due to a lack of coenzyme A, but to the acetyl radical.

In order to prove this, we followed what happened if the rats having been fed diets III and IV were given corrective diets.

C. The influence of corrective diet on acetylation indexes lowered by hepatic steatosis:

1. Action of sodium acetate. If the animals show a great lowering of the acetylation index due to the ingestion of diets II and III, are simultaneously given 1 mg of p-aminobenzoic acid of 0.5 millimole of sodium acetate per 100 g of body weight, the acetylation index is raised by approximately 20% as compared to those animals receiving only p-aminobenzoic acid. This fact thus supports the idea that it is indeed the acetyl radicals which are lacking in the rat organism subjected to a steatogenic diet in order to acetylate p-aminobenzoic acid.

2. Influence of the corrective diet. Steatosis due to a protein deficiency is reversible if choline is given as a lipotropic substance, and therefore we were naturally led to examine the effects of the addition of the this substance in the diet on the acetylation indexes of rats fed diets III and IV. The experiment was conducted in the following manner: 30 rats receiving diets II, III and IV. On the 14th day, the p-aminobenzoic acid acetylation is determined in urine; it was an average 57, 39, 32 respectively; in other words essentially comparable to that of the animals shown in table II. Each group was then separated into two lots--one receiving normal well-balanced diet number 1, the other receiving diets III and IV to which was added choline in amounts of 300 g of choline per 100 g of diet. The results obtained are shown in table II. The results being very explicit we calculated only the averages.

This table also includes the average values for the indexes of octanoic acid oxidation (not corrected for lipids) and the average amounts of total lipids in the liver. Examination shows that ingestion of either a steatogenic diet to which choline was added lasting 7 days, or a normal diet lasting 14 days, improves both the degree of acetylation and the oxidation indexes. There is also a raising of the octanoate oxidation indexes, however steatosis persists in some lots. The oxidation index reaches its normal level while the degree of acetylation is still 20 to 30% lower than normal; the similarity of the phenomena is thus less clear here than during the development of fatty livers. If octanoate oxidation is a reflection of the oxidation of higher fatty acids by the hepatic tissue, it must be admitted

that after a period of unavailability, the acetyl radicals are newly formed in abundance from fatty acids, but the organism prefers to use them for other purposes than the acetylation of p-aminobenzoic acid. This explanation is made more plausible by the large number of fundamental reactions in which the acetyl radicals are used.

Let us finally point out that choline, a lipotropic agent used in these kinds of fatty livers, increases the degree of acetylation more quickly than the ingestion of a normal diet.

CONCLUSION

Low protein diets give a large decrease in the degree of acetylation due to an inhibition of the oxidation of fatty acids which means a fall in the octanoic acid oxidation *in vitro* and steatosis. The ingestion of choline, an active lipotropic agent, brings about an increase of the degree of acetyation, but parallelism to octanoic acid oxidation and the amount of lipids in the liver is less clear than with the developement of steatosis, the acetyl radicals seemingly being after a long period of unavaliability, used for purposes other than p-aminobenzoic acid acetylation.

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L'acétylation de l'acide *p*-aminobenzoïque chez le Rat porteur de foie gras.

par LE VAN HUNG,

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Différentes hypothèses ont été faites sur l'origine des radicaux acétyle dans l'organisme reposant sur toute une série de recherches biochimiques faites à ce sujet. Une des grandes sources serait, à côté du pyruvate, les acides gras supé-

rieurs dont l'oxydation livre des chaînons à deux atomes de carbone générateurs d'acétyle : une série de travaux faits avec du C_{14} viennent à l'appui de cette façon de voir.

Ayant poursuivi un ensemble de recherches sur l'acétylation de l'acide *p*-aminobenzoïque *in vivo* chez le Rat, nous avons montré que le taux d'acétylation dans l'urine de cette substance, mesuré dans certaines conditions que nous avons définies (LE VAN HUNG, 1953), permet une mesure de l'activité acétylante de l'organisme.

Par ailleurs, l'étude du foie gras obtenu par un régime carencé en protéines a montré que la stéatose relève en partie d'une non-oxydation des acides gras (voir M. LÉVY, 1949). Nous avons institué des expériences sur le type suivant :

Des rats Wistar adultes recevaient un des 4 régimes ci-dessous :

Régime I normal contenant 20 p. 100 de caséine, 13 p. 100 de saindoux, 60 p. 100 de saccharose.

Régime II riche en caséine, contenant 80 p. 100 de caséine, 13 p. 100 de saindoux et pas de saccharose.

Régime III enrichi en lipides, contenant 20 p. 100 de caséine, 60 p. 100 de saindoux et 10 p. 100 de saccharose.

Régime IV sans caséine et contenant seulement 13 p. 100 de saindoux et 86 p. 100 de saccharose.

Tous les régimes contenaient en outre 5 p. 100 du mélange salin de McCARRISON et 2 p. 100 d'aliment encombrant. Les rats recevaient tous par jour et animal les quantités utiles de vitamines (selon GUILLAUME).

Le régime riche en caséine (II) possède une valeur énergétique de 1,400 calories pour 100 g de régime. Le régime III semble, *a priori*, contenir la même proportion de caséine que le régime I, car 100 g du régime normal ont une valeur énergétique de 437 calories, alors que 100 g du régime III en contiennent 660, si bien que les animaux ingèrent des quantités de caséine qui sont entre elles comme 457 — 206 pour une même quantité de calories.

A. *Expériences et résultats obtenus.*

Des rats pesant de 170 g à 200 g sont soumis à ces régimes durant 28 jours à raison de 7 animaux par régime. On détermine sur les 28 rats le taux d'acétylation de l'acide *p*-aminobenzoïque, avant le régime, puis les 7e, 14e, 21e et 28e jours suivant l'ingestion. On sacrifie les animaux immédiatement après et on prélève le foie. Une partie aliquote est broyée et sert à déterminer l'indice d'oxydation de l'acide octanoïque par la méthode de LEHNINGER et KENNEDY, le reste sert au dosage des lipides totaux.

Les résultats sont rassemblés dans le tableau I.

Étant donnée la haute teneur en lipides des foies dans les séries III et IV, nous avons calculé l'indice d'oxydation de l'acide octanoïque par gramme de foie délipidé.

L'examen du tableau I montre :

1° Que le régime riche en caséine n'apporte aucune modification au taux d'acétylation, non plus qu'à l'indice d'oxydation de l'acide octanoïque. Ce régime ne donne par ailleurs qu'une faible augmentation de la teneur en lipides totaux (4,14 100).

2° Par contre, les régimes III et IV apportent de grosses perturbations.

Le foie des animaux ayant reçu le régime riche en graisse et appauvri en caséine (III) (33 p. 100 de moins que dans le régime normal par 100 calories)

TABLEAU I. — *Composition des régimes*

Régime	Calories par kilo	Nature du lipide	Lipides en % p. 100	Indice d'acétylation — Au départ	7e j.	14e j.	21e j.	28e j.	Indice d'oxydation — Au départ	7e j.	14e j.	21e j.	28e j.
1. Normal	4,77	Normal	6,7	63 ± 3	63 ± 3	59 ± 4	61 ± 2	64 ± 3	55	62	[illegible]	[illegible]	70
2. Riche en caséine	14	Normal	7,7	60 ± 3	57 ± 1	56 ± 4	55 ± 5	57 ± 4	[illegible]	[illegible]	[illegible]	[illegible]	40
3. Riche en graisse	3,05	Très gras	22	60 ± 4	47 ± 4	42 ± 3	36 ± 3	37 ± 4	[illegible]	[illegible]	[illegible]	[illegible]	18
4. Sans caséine	4	Très gras	20	61 ± 3	60 ± 3	53 ± 4	33 ± 2	17 ± 3	10	5	[illegible]	[illegible]	13

TABLEAU II. — *Taux d'acétylation de l'acide paraminobenzoïque, teneur en lipides totaux et indice d'oxydation de l'acide octanoïque du tissu hépatique chez les rats ayant reçu successivement les régimes II, III, IV, puis revenus au régime normal.*

N° de régime	14e jour après le régime déséquilibré — II	III	IV	7e jour après le même régime + choline — II	III	IV	14e jour après le régime normal — II	III	IV
Taux d'acétylation		39 ± 1	32 ± 3		48 ± 1	47 ± 3	57	56	42 ± 3
Indice d'oxydation de l'acide octanoïque	2,86 ± 0,24	1,19 ± 0,31	1,05 ± 0,15	—	2,35 ± 0,25	2,24 ± 0,20	2,57	2,57 ± 0,17	3,00 ± 0,16
Teneur hépatique en lipides totaux sur le poids frais	7,7	32	29		8,4	17		[illegible]	[illegible]

... gras, contenant environ 5 fois plus de lipides totaux que le foie normal. L'indice d'acétylation de ces animaux est abaissé, dès le 7e jour, de 21 p. 100 ... valeur; cette chute s'accentue ensuite mais plus lentement, elle est de ... p. 100 au 28e jour. A ce moment l'indice d'oxydation de l'acide octanoïque est inférieur de 57 p. 100 à l'indice normal, de 40 p. 100 si l'on prend en considération les indices corrigés. Donc la stéatose s'accompagne d'une chute importante et du même ordre, du pouvoir d'acétylation de l'acide *p*-aminobenzoïque et d'oxydation de l'acide octanoïque.

En ce qui concerne le régime IV dépourvu de caséine, on voit des faits analogues, mais présentant des particularités. La surcharge en graisse est importante (90 p. 100) mais moins grande qu'avec le régime III; néanmoins, les deux systèmes enzymatiques sont ici plus touchés : l'indice d'acétylation est abaissé de 51 p. 100 au 7e jour, de 46 p. 100 au 21e jour et subit un effondrement au 28e jour à 72 p. 100; l'acétylation n'est plus que 28 p. 100 de la valeur normale, de même l'indice d'oxydation de l'acide octanoïque a ici abaissé de 62 p. 100 pour le foie frais et de 57 p. 100 pour l'indice corrigé du foie délipidé. Ici encore, le parallélisme entre les phénomènes est évident.

B. Il ressort de ces expériences que l'oxydation des acides gras se présente comme la grande source de radicaux servant à l'acétylation de l'acide *p*-aminobenzoïque. Le régime II riche en protéine n'entraînant pas de modification, ni dans l'acétylation, ni dans l'oxydation de l'acide octanoïque, donne une preuve, formelle que les protéines ne sont pas une source de radicaux acétyle, fait d'ailleurs déjà longtemps admis jusqu'ici. Par contre l'ingestion d'un régime pauvre en protéines ou exagérément riche en glycérides par rapport aux protéines, occasionne une chute importante de l'activité acétylante du foie.

Nous avons fait des expériences montrant que, si, à ces animaux présentant ce type de foie gras, on donne un excès d'acide pantothénique, il n'y a pas l'amélioration du taux d'acétylation. Donc la chute n'est pas due à un manque de coenzyme A mais bien de radicaux acétyle.

Pour le prouver, nous avons suivi ce qui se passe, si, à des rats ayant ingéré les régimes III et IV, on donne des régimes correcteurs.

C. *Influence des régimes correcteurs sur le taux d'acétylation abaissé par stéatose hépatique.*

1° *Action de l'acétate de sodium.*

Si à des animaux présentant du fait de l'ingestion des régimes II et III un fort abaissement du taux d'acétylation, on donne simultanément 1 mg d'acide *p*-aminobenzoïque et 0,5 millimolécule d'acétate de sodium par 100 g de poids vif, on voit que le taux d'acétylation subit un relèvement de 20 p. 100 environ par rapport à celui des animaux recevant de l'acide *p*-aminobenzoïque seul. Ce fait témoigne en faveur de l'idée que ce sont bien les radicaux acétyle qui manquent à l'organisme du rat soumis au régime stéatogène pour acétyler l'acide *p*-aminobenzoïque.

2° *Influence du régime correcteur.*

Les stéatoses dues à une déficience en protéines étant réversibles si l'on donne une substance lipotrope, de la choline, nous avons naturellement amené à examiner les effets de l'adjonction de cette substance au régime, sur les taux d'acétylation des rats ayant été nourris avec les régimes III et IV. L'expérience a été menée de la façon suivante : 30 rats reçoivent les régimes II, III, IV. Au 14e jour, on détermine leur taux d'acétylation de l'acide *p*-aminobenzoïque dans l'urine; il est respectivement 57, 39, 32 en moyenne, c'est-à-dire très sensiblement semblable à celui des animaux correspondants au tableau II. Chaque groupe est alors séparé en deux lots. Un reçoit le régime normal n° 1 bien équili-

bre l'autre les régimes III, IV additionnés de choline à raison de 300 mg de choline pour 100 g de régime. On obtient les résultats mentionnés dans le tableau II. Les résultats étant très cohérents, nous n'avons fait figurer que des moyennes.

Ce tableau comporte également les valeurs moyennes des indices d'oxydation de l'acide octanoïque (non corrigées pour les lipides) et les teneurs moyennes du foie en lipides totaux. Son examen montre que l'ingestion, soit du régime stéatogène additionné de choline durant 7 jours, soit d'un régime normal durant 14 jours améliore les taux d'acétylation et les indices d'oxydation. Il y a un relèvement également des indices d'oxydation de l'octanoate, cependant que la stéatose persiste dans certains lots. L'indice d'oxydation a repris sa valeur alors que le taux d'acétylation est encore de 20 à 30 p. 100 inférieur à la normale, le parallélisme des phénomènes est donc ici moins net qu'au cours de l'installation du foie gras. Si l'oxydation de l'octanoate est un reflet de l'oxydation des acides gras supérieurs par le tissu hépatique, il faudrait admettre qu'après la période de carence les radicaux acétyle sont à nouveau formés en abondance à partir des acides gras, mais que l'organisme les utilise préférentiellement pour d'autres buts que l'acétylation de l'acide *p*-aminobenzoïque. Cette explication est rendue plausible par le grand nombre de réactions fondamentales en vue desquelles les radicaux acétyle sont utilisés.

Remarquons enfin que la choline, lipotrope appropriée à nos types de foie gras, améliore plus vite le taux d'acétylation que l'ingestion du régime normal.

Conclusion.

Les régimes pauvres en protéines donnent une forte diminution du taux d'acétylation due à une inhibition de l'oxydation des acides gras que traduisent la chute de l'indice d'oxydation de l'acide octanoïque *in vitro* et la stéatose. L'ingestion de choline, lipotrope actif, permet un relèvement du taux d'acétylation, mais le parallélisme avec l'oxydation de l'acide octanoïque et la teneur du foie en lipide est moins net qu'au cours de l'installation de la stéatose, les radicaux acétyle étant vraisemblablement, après une longue période de déficit, mobilisés en vue d'autres buts que l'acétylation de l'acide *p*-aminobenzoïque.

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Effect of Sodium Acetate on Tm_{PAH} Depressed by Exposure to Hydrazine

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Dogs were exposed to 0.63 mM/kg of hydrazine. They were treated 2 hours later with sodium acetate, alpha-lipoic acid, or alpha-lipoamide. Exposure to hydrazine produced an expected marked decrease in Tm_{PAH}. The infusion of the hydrazine-treated dogs with sodium acetate resulted in a transient increase in Tm_{PAH}. Some sparing effect of alpha-lipoamide on the PAH transport mechanism inhibited by hydrazine was suggested, although the magnitude of the sparing effect was not statistically significant.

WONG[6] AND VAN STEE[7] have shown that exposure of dogs to subconvulsigenic doses of hydrazine causes a decrease in the ability of the kidney to reabsorb glucose and clear para-aminohippurate (PAH), respectively. The present paper reports the results of an experiment to further elucidate the mechanism of PAH transport in which dogs with hydrazine-depressed Tm_{PAH} were treated with acetate, 6,8-epidithioctanoate (alpha-lipoate), and 6,8-epidithioctanoamide (alpha-lipoamide).

MATERIALS AND METHODS

Mature, female, mongrel dogs from 9 to 17 kg were used. The dogs were free from obvious systemic diseases and defects. They were fasted for 24 hours prior to use. The dogs were anesthetized and maintained throughout the experiment with a solution of pentobarbital sodium (50 mg/ml) given intravenously.

Polyethylene catheters were placed in a femoral vein for infusion and femoral artery for blood collection. Urine was collected through an indwelling Foley catheter.

A solution containing 5 gm/liter of inulin and 10 gm/liter of para-aminohippuric acid was adjusted to a pH of 7.0 to 7.5 with 1.0 N sodium hydroxide solution. A priming dose of 1.5 ml/kg of body weight of the infusion solution was given intravenously followed by a metered infusion of the same solution at a rate of

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0.20 ml/min/kg. This produced plasma levels of PAH from 150 to 500 μg/ml. Within this range of plasma PAH levels, the renal proximal tubular maximum for PAH (Tm_{PAH}) was maintained. Plasma amino levels were maintained between 200 and 800 μg/ml. Following priming the dogs were allowed a 45 to 60-minute equilibration prior to the first sampling. Throughout the infusion and sampling periods a separate intravenous infusion of 5 per cent dextrose in water was maintained to promote a mild water diuresis.

Four consecutive 30-minute determinations of Tm_{PAH} were performed on the dogs immediately prior to their use in the experiment. Only dogs which had an average Tm_{PAH} more than 300 μg/min/kg during any of the 30-minute periods were used. Our previous experience has shown that nearly all dogs with a degree of functional impairment reflected in a Tm_{PAH} below 300 μg/min/kg will have a significant degree of renal pathology.

Reagent grade anhydrous hydrazine (95 per cent, Matheson Coleman and Bell) was used in preparing the 0.9 per cent sodium chloride-hydrazine solutions for exposure of the animals. The acetate solution contained 10 per cent (w/v) sodium acetate adjusted to pH 7.0 to 7.5 with 1.0 N hydrochloric acid. The alpha-lipoic acid was dissolved in 3 ml of 1.0 N sodium hydroxide solution and diluted to 150 ml with 0.9 per cent sodium chloride solution. The alpha-lipoamide was dissolved in less than 10 ml of warm propylene glycol and diluted to 150 ml with 0.9 per cent sodium chloride solution.

The exposures to hydrazine were single intravenous injections of 20 mg (0.63 mM)/kg of body weight of hydrazine in several milliliters of 0.9 per cent sodium chloride solution. The 10 per cent sodium acetate solution was infused intravenously at a rate of 10 ml/hr/kg of body weight. The alpha-lipoic acid and alpha-lipoamide solutions contained 200 mg of the respective substances in 150 ml of solution which was infused at a rate of 50 ml/hr. The propylene glycol solution (lipoamide vehicle) contained 10 ml of propylene glycol to which was added an equal volume of 0.9 per cent sodium chloride solution. It was given by rapid single injection.

PAH concentration in the urine and plasma was determined according to the method of Bratton and Marshall modified by Smith et al. Slight modification of the PAH method was required to accomplish the determination in the presence of minute traces of hydrazine. Using a half-saturated aqueous solution of benzaldehyde in place of water to prepare the hydrochloric acid reagent allowed the color formation to proceed in the presence of trace amounts of hydrazine in the plasma or urine. Inulin concentrations in the urine and plasma were determined by the resorcinol method of Little as modified by Smith. The presence of trace amounts of hydrazine did not interfere with the inulin determination.

Consecutive 30-minute determinations of Tm_{PAH} were performed for six hours on 2 separate groups of untreated controls. The 4 dogs treated with hydrazine only were run for 2 hours followed by exposure. Eight 30-minute determinations were made following exposure. The dogs treated with sodium acetate, propylene glycol (alpha-lipoamide vehicle), and alpha-lipoic acid only were run similarly except that these substances were given by sustained intravenous infusion rather than by rapid, single injection. A group treated with alpha-lipoamide only was not run because of the limited availability of alpha-lipoamide.

Three groups of dogs treated with hydrazine were given the metabolic intermediates described. Each group was run for 2 hours before any treatment, exposed to 0.63 mM/kg of body weight of hydrazine and run for 2 more hours to obtain significant depression of tubular function. Following this initial 4-hour preparation sustained infusion of the metabolic intermediates was continued for 3 more hours in a manner similar to their respective control groups. The hydrazine-plus-acetate-treated group contained 3 dogs, the hydrazine-plus-alpha-lipoic acid-treated group, 3 dogs, and the hydrazine-plus-alpha-lipoamide-treated group, 3 dogs.

Analysis of the data was by means of the technic of repeated measurement analysis of covariance.[4]

RESULTS

The hourly means adjusted for the average of the two pre-exposure values (covariance technic) for the controls and the hydrazine-treated groups are plotted in Figure 1. The values for hours 1 and 2 are means

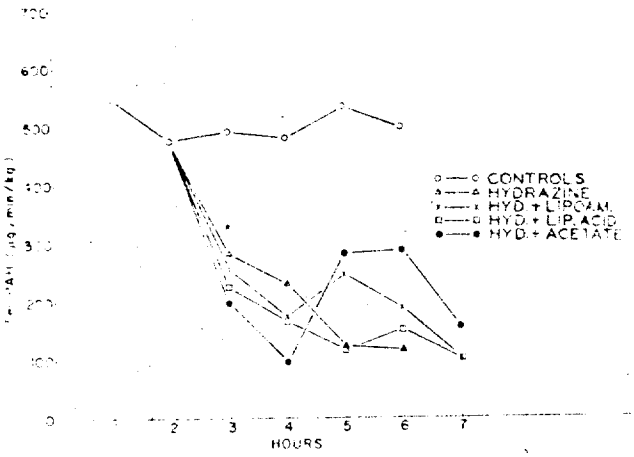

Fig. 1. Adjusted hourly mean (see text) Tm_{PAH} following exposure to hydrazine with subsequent treatment with various metabolic intermediates.

for all of the groups prior to exposure. The plot of the control data (hours 3-6) represents an average of the adjusted means of the two control groups, one of which was run early in the experiment and the other of which was run late in the experiment. The hydrazine data represent the group treated with hydrazine but not treated with a metabolic intermediate. The remaining three lines represent data from the groups treated with hydrazine and sodium acetate, hydrazine and alpha-lipoic acid, and hydrazine and alpha-lipoamide, respectively. All hydrazine exposures were made at the

end of hour 2 and the metabolic intermediate exposures were made at the end of hour 4.

The adjusted group means for the individual control groups and the groups treated with sodium acetate and lipoic acid, respectively, but not with hydrazine, are given in Table I. No significant difference was found

TABLE I. CONTROL, LIPOIC ACID, AND SODIUM ACETATE ADJUSTED GROUP MEANS (HOURS 3-5)

Group	N	AM
Control-I	6	497
Control-II	4	458
Lipoic Acid	3	498
Sodium Acetate	3	722

N Number of samples.
AM Adjusted mean Tm_{PAH} (μg/min/kg)

between the two control groups, the controls and the group treated with lipoic acid only, and the controls and the group treated with the propylene glycol vehicle. The values for the group treated with sodium acetate only were significantly higher than the controls ($P < 0.05$).

The group treated with hydrazine only differed significantly from the controls during hour 3 ($P < 0.05$) and during hours 4, 5, and 6 ($P < 0.01$). The groups treated with hydrazine followed by the various metabolic intermediates did not vary significantly at each hour from the group treated with hydrazine only. However, the adjusted mean for the group (hydrazine plus sodium acetate) increased from 95 at hour 4 to 281 at hour 5, while the adjusted means for the group (hydrazine only) dropped from 228 to 121 during this time. The increase is significantly different from the decrease at the 0.01 level even though the levels themselves did not vary significantly. Although similar, but smaller, "humps" occur in the plots of the other two hydrazine groups treated with metabolic intermediates, they were not statistically significant.

DISCUSSION

The well-known phenomenon of the augmentation of the Tm_{PAH} by the infusion of sodium acetate was demonstrated (Table I). Following exposure to hydrazine, infusion of acetate resulted in a sharp increase of Tm_{PAH} (Figure 1) which, although significantly above the preinfusion level, was still significantly below the control level. Schachter, et al.[2] have suggested that the PAH transport mechanism is normally partially occupied by acylglycine derivatives and that the stimulating effect of acetate is attributable to its ability to eliminate these competitive inhibitors. Although the mechanism of inhibition is not yet known, the effectiveness of hydrazine in very small quantities, *in vivo*, would suggest that it is not competitive but rather, uncompetitive. Such a system would have sites successively inactivated by binding with hydrazine so that at any given instant both active and inactive sites would be present. The active sites would be functional, partially occupied by acylglycine derivatives, and hence amenable to the stimulating effects of acetate. A system uncompetitively inhibited by hydrazine would not be affected by acetate, as these observations suggest that this one is not. Such a system would respond to acetate infusion with a transitory rise in Tm_{PAH} which would decline as the hydrazine progressively inactivated and the remaining active sites of the transport mechanism.

Although the fact was not statistically significant and increase of Tm_{PAH} which may have occurred following the treatment of the hydrazine-treated dogs with alpha-lipoamide (Figure 1) is suggestive of some sparing effect of the metabolic intermediate on the ability of the nephron to transport PAH in the presence of hydrazine. The implications of such a phenomenon are unclear.

SUMMARY

Dogs were exposed to 0.63 mM/kg of hydrazine. They were treated 2 hours later with sodium acetate, alpha-lipoic acid, or alpha-lipoamide. Exposure to hydrazine produced an expected sharp decrease in Tm_{PAH}. The infusion of the hydrazine-treated dogs with sodium acetate resulted in a transient increase in Tm_{PAH}. Some sparing effect of alpha-lipoamide on the PAH transport mechanism inhibited by hydrazine was suggested, although the magnitude of the effect was not statistically significant.

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Bull. du Cancer, 54(4):359-362, 1965

Results of a New Series of Experiments on the Prevention of Experimental Cancer

by Jean Verne and Paul C.-J. Roth
with the technical assistance of M. Strazielle

The series which we had the honor to present to you last year (1), involved experiments done on fenale mice of R. III and C_3H breeds, having received under the dorsal skin an implantation of 2/10 mg of crystallized dihydrofolliculin and then treated with subcutaneous injections of norandrostenolone phenpropionate (Durabolin), 8 to 10 days after implantation. It will be recalled that this substance possesses anabolic properties which act on protein anabolism and indirectly, on calcium anabolism. Its retention of calcium as well as phosphorus and potassium is in our opinion of prime importance.

In this new series, we used only mice of the C_3H breed which are quite receptive to carcinogenesis, while mice of R. III breed are not quite as receptive. Further, we experimented on mice of both sexes, but gave to the males a different preventive treatment, consisting of injections of Lutogyl.

As in our previous experiments, we subjected the animals to two different diets:

a) diet acidulated by 2.5 cm^3/l of CH_3COOH (pH 4.5) or

b) diet of tap water (pH 7.5), diet said to be of "ordinary water".

(1) Bull. Assn. Franc. Cancer, 51: 287-290, 1964

We did not set up in this series a diet strongly alkalinized by 12.5 g/l of CO_3NaH (pH 8.5), which we previously showed was an accelerator of carcinogenesis.

Material and Methods

We used in this series, 113 females and 14 males of the C_3H breed, all receiving an implant of 2/10 of a milligram of dihydrofolliculin, and 7 females who received two implants of the same posology, making a total of 134 mice.

Of these animals, 98 females were treated with 10 to 11 weekly injections of Durabolin, so that the total amount of the ester injected reached to 2 to 2.2 mg. Also, 22 females received no treatment. The 14 males received 13 mg of Lutogyl in 11 subcutaneous injections. Further, 106 of the females and the 14 males were given a diet of acidulated liquid, while 14 untreated females of the 22 had a diet of ordinary water.

Results

Out of the 98 treated females, 12 tumors appeared.

Of 8 untreated females, one tumor appeared. (These 106 females were subjected to the diet of acidulated water, pH 4.5).

Of 7 untreated females, subjected to the diet of ordinary water (pH 7.5), we found 5 tumors.

The 7 untreated females who drank ordinary water and who recieved a second implant all developed tumors.

Of the 14 males receiving implants and drinking acidulated water (pH 4.5) and treated with Lutogyl, only one tumor appeared.

Therefore out of 120 mice (106 females and 14 males) implanted and treated, some with 2 to 2.2 mg Durabolin, and others with 13 mg Lutogyl (6 times the dose of Durabolin), we verified the appearance of 14 tumors, which gave a percentage of 11.66 p. 100. In the preceding series, the percentage was only 6.8 p. 100. This difference came from the presence, in that series composed of 175 mice, of 99 females of the R. III breed in which no tumors appeared whether they were treated or not with Durabolin. Therefore, the 12 tumors verified in that series all appeared in the 76 females of the C_3H breed, which makes a percentage of tumors relating only to the mice of this breed of 15.78 p. 100.

There is therefore an improvement since the percentage in this present series is 11.66 p. 100, and this may be attributed to the amount of Durabolin, which was generally higher than the amounts used previously.

Further, we treated three tumors with intra-tumoral injections of 6 to 14 mg of Endoxan, which had the effect of reabsorbing these tumors. Several other mice, treated with the same product died, the tumors being in a more advanced stage.

Comments

Lutogyl is therefore shown to be effective in the neutralization of dihydrofolliculin, since we verified the presence of only one tumor out of 14 treated males.

Since 1935, Lacassagne has been trying to find hormones which can oppose the carcinogenic effect of folliculin. He published his first results in 1937 obtained with progesterone and testosterone, and in

1939, he published with A. Raynaud new results obtained on the influence of estrone on male mice, but in alternation with injections of testosterone propionate. The treatment lasted six months and nevertheless, carcinomas appeared in some males; this same thing occurred in mice of both sexes, in which the amount of testosterone alternated with 50 gammas of estrone, was brought to 2.5 mg per week, which was 50 times stronger than the amount of female hormone.

In our experiment on males, it was by contrast, the amount of Lutogyl which was 6 times higher in the same time as the amount of Durabolin administered to the females. We consider this experiment to be encouraging, and we expect to experiment on mice of both sexes to study the preservative influence of synthetic progestogens.

Conclusion

In this series, the preventive power of norandrostenolone phenpropionate in the appearance of cancer was confirmed experimentally by the implantation of crystalline compounds of dihydrofolliculin, because out 7 untreated females (subjected to a diet of slightly alkaline water), there appeared 5 tumors.

Further, in males implanted in the same way with the same hormone, subjected to a diet of acidulated water and treated with injections of Lutogyl, there appeared only one tumor.

This last experiment opens interesting perspectives on the neutralization of the carcinogenic effects of dihydrofolliculin, in mice of both sexes.

Work from the laboratory of Prof. Verne at the Faculty of Medicine of Paris and from the Ethology Laboratory of Wild Animals of the Museum of Natural History.

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Résultats d'une nouvelle série d'expériences sur la prévention d'un cancer expérimental

par

Jean VERNE et Paul C.-J. ROTH

avec l'assistance technique de

M. STRAZIELLE

Dans les séries que nous eûmes l'honneur de vous présenter l'an dernier (1), il s'agissait d'expériences faites sur des souris femelles des lignées R. III et C_3H, ayant reçu, sous la peau dorsale, une implantation de 2/10 de mg de di-hydro-folliculine cristallisée et traitées, ensuite, par des injections sous-cutanées de *Phényl-propionate de nor-Androsténolone* (*Durabolin*), 8 à 10 jours après l'implantation. Ainsi que nous l'avons déjà rappelé, cette substance possède des propriétés anabolisantes, qui agissent sur l'anabolisme protidique et, indirectement, sur l'anabolisme calcique. Son action de retenue du Calcium est, à notre avis capitale, ainsi que celle du Phosphore et du Potassium.

Dans cette nouvelle série, nous avons uniquement expérimenté sur des souris de la lignée C_3H qui ont conservé une assez vive réceptivité à la cancérisation, alors que les souris de la lignée R. III l'ont à peu près perdue. De plus, nous avons expérimenté sur des souris des deux sexes, mais en appliquant, aux mâles, un traitement préventif différent, puisqu'il s'agissait d'injections de *Lutogyl*.

(1) *Bull. Ass. franç. Cancer*, 1964, t. 51, pp. 287-290.

Comme lors de nos essais antérieurs, nous avons soumis les animaux à des régimes différents :

a) régime acidulé par 2,5 cm³/l de CH_3COOH (pH 4.5) ou

b) régime de l'eau de distribution (pH 7.5), régime dit « de l'eau ordinaire ».

Nous n'avons pas instauré, dans cette série, de régime fortement alcalinisé par 12.5 g/l de CO_3 NaH (pH 8,5), qui nous a antérieurement montré qu'il était un accélérateur de la cancérisation.

Matériel et méthodes

Nous avons utilisé, dans cette série, 113 femelles et 14 mâles de la lignée C_3H qui reçurent, toutes et tous, un implant de 2/10 de milligramme de di-hydrofolliculine, et 7 femelles qui reçurent *deux* implants de même posologie soit, au total : 134 souris.

De ces animaux, 98 femelles furent traitées par 10 à 11 injections hebdomadaires de Durabolin, de manière à ce que le total de l'ester injecté atteigne 2 à 2,2 mg. D'autre part, 22 femelles ne furent pas traitées. Les 14 mâles reçurent 13 mg de Lutogyl en 11 injections sous-cutanées. En outre, 106 des femelles et les 14 mâles furent soumis au régime de la boisson acidulée, alors que 14 femelles non traitées, sur 22, furent soumises au régime de l'eau ordinaire.

Résultats

Sur 98 femelles traitées, il apparut 12 tumeurs.

Sur les 8 femelles non traitées, il apparut une tumeur. (Ces 106 femelles avaient été soumises au régime de l'eau acidulée, pH 4.5).

Chez les 7 femelles non traitées, soumises au régime de l'eau ordinaire (pH 7.5), nous constatâmes 5 tumeurs.

Les 7 femelles non traitées, qui buvaient de l'eau ordinaire et qui avaient reçu un deuxième implant, *furent toutes tumorisées*.

Chez les 14 mâles implantés, buvant de l'eau acidulée (pH 4,5) et traités par le Lutogyl, il ne survint *qu'une seule tumeur*.

Donc, sur 120 souris (106 femelles et 14 mâles) implantées et traitées, les unes par 2 à 2,2 mg de Durabolin et, les autres, par 13 mg de Lutogyl (6 fois la dose de Durabolin), nous avons constaté l'apparition de 14 tumeurs, *ce qui donne un pourcentage de 11,66 p. 100*. Dans la série précédente, le pourcentage n'était que de 6,8 p. 100. Cette différence provient de la présence, dans cette série composée de 175 souris, de 99 femelles de la lignée R. III, chez lesquelles il n'apparut aucune tumeur, qu'elles aient été traitées ou non par le Durabolin. Donc, les 12 tumeurs constatées dans cette série apparurent, toutes, sur les 76 femelles de la lignée C_3H,

ce qui fait que le pourcentage de tumeurs ne concernait que les souris de cette lignée, ce qui aurait donné 15.78 p. 100 de tumeurs.

Il y a donc une amélioration, puisque le pourcentage dans cette présente série est de 11,66 p. 100, ce qui peut être imputé à la dose de Durabolin qui fut, en général supérieure aux doses utilisées précédemment.

D'autre part, nous avons traité trois tumeurs par des injections, intra-tumorales, de 6 à 14/10 de mg d'Endoxan, qui firent se résorber ces tumeurs. Plusieurs autres souris, traitées par ce même produit moururent, les tumeurs étant à un stade plus avancé.

Commentaires

Le Lutogyl s'est donc montré effectif dans la neutralisation de la di-hydro-folliculine, puisque nous n'avons constaté que la présence d'une seule tumeur sur les 14 mâles traités.

Dès 1935, Lacassagne avait entrepris de rechercher quelles hormones pourraient s'opposer à l'action carcinogène de la folliculine. Il publia ses premiers résultats en 1937 obtenus avec la progestérone et la testostérone et, en 1939, il publia avec A. Raynaud de nouveaux résultats obtenus sur l'influence de l'œstrone chez des souris mâles, mais en alternance avec des injections de propionate de testostérone. « Le traitement dura 6 mois, écrivait-il, et des carcinomes apparurent néanmoins chez certains de ces mâles, ce même phénomène se reproduisit chez des souris des deux sexes, chez qui la dose de testostérone, alternée avec 50 gammas d'œstrone, fut portée à 2,5 mg par semaine, soit 50 fois plus forte que celle de l'hormone femelle. »

Dans notre essai sur des mâles ce fut, au contraire, la dose de Lutogyl qui fut 6 fois plus élevée, dans le même temps, que la dose de Durabolin administrée aux femelles. Nous considérons cet essai comme encourageant et nous comptons expérimenter, sur des souris des deux sexes, l'influence préservatrice des progestatifs de synthèse.

CONCLUSION

Dans cette série, le phényl-propionate de nor-androsténolone a confirmé son *pouvoir préventif* de l'apparition d'un cancer, expérimentalement provoqué par l'implantation de comprimés cristallins de di-hydro-folliculine, car sur 7 femelles *non traitées* (soumises au régime de l'eau légèrement alcaline), il apparut 5 tumeurs.

D'autre part, chez les mâles également implantés avec la même hormone, soumis au régime de l'eau acidulée et traités par l'injection de Lutogyl, il n'apparut qu'une seule tumeur.

Ce dernier essai ouvre des perspectives intéressantes sur la neutralisation des effets cancinogènes de la di-hydro-folliculine, chez les souris des deux sexes.

Travail du Laboratoire du Pr VERNE *à la Faculté de Médecine de Paris et du Laboratoire d'Ethologie des Animaux sauvages du Muséum National d'Histoire Naturelle.*

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THE EFFECT OF ACETATE, PYRUVATE, AND GLUCOSE ON ALCOHOL METABOLISM*

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Although many investigators have studied the effects of pyruvate and glucose on alcohol metabolism, there is still disagreement as to what effect they have on the rate of alcohol oxidation. Jacobsen (1) discussed the various reports in which the results vary from no effect of pyruvate, inhibition by pyruvate or glucose, or an actual increase in the rate of alcohol oxidation by pyruvate or glucose.

Bartlett and Barnet (2), using radioactive ethanol, concluded that alcohol metabolism was markedly inhibited by pyruvate. Only one animal was used, however, and only one level of alcohol studied. A more extensive study by the tracer technique has therefore been done with both acetate and pyruvate. Acetate was used since its oxidation involves the same metabolic pathways as pyruvate through the tricarboxylic acid cycle. Thiamine-deficient animals were included, since pyruvate oxidation should be partially blocked under these conditions. The effect of glucose on blood alcohol disappearance in human beings was also studied.

EXPERIMENTAL

Rat Studies.—Young adult female rats from the Charles River Breeding Laboratories, Inc., weighing approximately 200 gm. were used. The control rats were fed Purina lab chow *ad libitum*. Other rats were maintained on a thiamine free diet until they had lost approximately 25 per cent of their body weight. At that time they were fed the basal diet (Table I) *ad libitum* twice a week in order to keep them alive and chronically deficient.

The rats were given various amounts of 20 per cent ethanol-1-C^{14} intraperitoneally. 2 hours after the alcohol had been administered, the sodium pyruvate or acetate was administered intraperitoneally at a level of 20 mM per kilo of body weight. In the experiments with thiamine-deficient

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animals 2 gm. of alcohol per kilo of body weight were given with and without pyruvate. The effect of acetate and pyruvate on alcohol metabolism was also studied in a small group of control animals which had received daily subcutaneous injections of 20 mg. of niacin per kilo of body weight for 5 days prior to the study.

When acetate or pyruvate was given, the animals were quickly removed from the respirometer, injected with the appropriate metabolite, and immediately put back into their chambers.

Expired CO_2 was collected and radioactivity determined as previously reported (4).

TABLE I

Basal Diet

Constituents	
	per cent
Casein, purified	20.0
Glucose	70.7
Lard	4.0
Salts 4 (Hogsted *et al.* (3))	4.0
Cod Liver oil	1.0
Choline chloride	0.3
	mg. per 100 gm. diet
Thiamine chloride	0.4
Riboflavin	0.8
Calcium pantothenate	2.5
Pyridoxine hydrochloride	0.4
Niacin	4.0

Human Studies—The human subjects used in these studies were patients at the Long Island Hospital, Boston, Massachusetts. Most of these patients were chronic alcoholics. They were given whisky orally to provide 0.5 or 1 gm. of alcohol per kilo of body weight. Blood samples for alcohol determinations were taken every hour for approximately 5 hours. On a second occasion these same subjects were given the same dose of alcohol and 100 gm. of sucrose were fed 2 hours after they had been given the alcohol. In these experiments determinations of both blood alcohol and blood sugar were made.

Alcohol was determined by the method of Gibson and Blotner (5) with slight modifications. The alcohol was distilled directly from whole blood into the potassium dichromate solution. A Beckman DU spectrophotometer was used instead of a colorimeter. Blood glucose was determined by the method of Somogyi (6).

Results

The effect of pyruvate or acetate administration upon the amount of alcohol oxidized at different dosage levels is shown in Table II. As has been previously reported (4), alcohol oxidation is maximal between 2 and 2.5 gm. of alcohol per kilo of body weight and gradually decreases at higher levels. At a dose of 2.0 to 2.5 gm. of alcohol approximately 80 per cent of

TABLE II

Effect of Acetate and Pyruvate on Alcohol Metabolism

Alcohol dose		Pyruvate (P.) or acetate (A.)	No. of rats	Alcohol metabolized in 7 hrs.		Difference
gm. per kg.	*mM per kg.*	*mM per kg.*		*per cent of dose*	*mM per kg.*	*mM per kg.*
2.0–2.5	43.4–54.3	0	15	80.7 ± 1.1*	39.4	
		20 (P.)	17	54.3 ± 3.1	25.4	14.0
		20 (A.)	4	53.5 ± 2.8	25.1	14.3
3.0	65.2	0	6	64.5 ± 0.5	42.1	
		20 (P.)	2	44.3, 53.2	25.2	16.9
4.0	86.8	0	2	45.4, 47.0	40.1	
		20 (P.)	2	29.4, 29.1	25.3	14.8
		20 (A.)	1	30.0	26.0	14.1

* Standard error of the mean.

TABLE III

Effect of Pyruvate on Alcohol Metabolism in Chronic Thiamine Deficiency

Two rats used in each experiment.

Alcohol dose		Pyruvate	Alcohol metabolized in 7 hrs.		Difference
gm. per kg.	*mM per kg.*	*mM per kg.*	*per cent of dose*	*mM per kg.*	*mM per kg.*
2.0	43.4	0	82.3, 80.7	34.5	
		20	67.6, 62.0	28.1	6.4

the activity in the alcohol was recovered in the expired CO_2 over the 7 hour period from control animals. The administration of pyruvate or acetate decreased the recovery of C^{14} in the expired CO_2 to approximately 54 per cent. When the results are expressed in terms of millimoles of alcohol metabolized per kilo of body weight, the difference between the control animals and those which received either acetate or pyruvate is approximately 14 mM, which is equivalent to 70 per cent of the acetate or pyruvate given.

As might be expected, in the thiamine-deficient rats in which the oxidation of pyruvate is limited, the effect of pyruvate was less marked (Table III). Alcohol oxidation remained essentially normal, approximately 35

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mM per kilo of body weight over the 7 hour period, but the decrease after pyruvate administration was only 6.4 mM compared to 14 mM in the control animals under comparable conditions.

That the effect of pyruvate was not simply to suppress $C^{14}O_2$ excretion but in fact to inhibit the disappearance of blood alcohol is seen in Table IV. Rats were given 2.5 gm. of alcohol per kilo of body weight intraperitoneally and 1 hour later blood was taken from the jugular vein for alcohol determinations. At this time half the animals were given 20 mM of

TABLE IV

Effect of Pyruvate on Blood Alcohol Disappearance

Eight animals used in each experiment.

Alcohol dose	Pyruvate	Decrease in blood alcohol concentration between 1 and 2 hrs.	Disappearance
gm. per kg.	*mM per kg.*	*mg. per cent*	*mg. per cent*
2.5	0	59.6 ± 8.5	
	20	31.0 ± 2.7	28.6*

* Statistically significant ($P < 0.05$).

TABLE V

Effect of Pyruvate and Acetate on Alcohol Metabolism in Niacin-Treated Rats*

Alcohol dose		Pyruvate (P.) or acetate (A.)	No. of rats	Alcohol metabolized in 6 hrs.		Difference
gm. per kg.	*mM per kg.*	*mM per kg.*		*per cent of dose*	*mM per kg.*	*mM per kg.*
2.0	43.4	0	5	74.0 ± 1.6	32.5	
		20 (P.)	4	71.8 ± 1.7	31.2	1.3
		20 (A.)	2	71.9, 65.4	29.8	2.7

* Animals injected daily with 20 mg. of niacinamide per kilo of body weight for 5 days.

pyruvate (Na salt) intraperitoneally. 1 hour later blood was again drawn from the jugular vein from both groups of animals. The fall in blood alcohol concentration was approximately 59 mg. per cent in those animals which did not receive pyruvate, whereas in the animals which received the pyruvate it was 30 mg. per cent.

Table V shows the results with niacin-treated rats. The inhibitory effect of acetate or pyruvate demonstrated in control animals was not observed after niacin treatment.

Fig. 1 illustrates the effect of orally administered sucrose on the disappearance of blood alcohol from four human subjects. In each case when

the levels of blood glucose rise, there is a marked decrease in the rate of alcohol disappearance, and, when the levels of blood sugar begin to fall, there is an increase in the rate of blood alcohol disappearance.

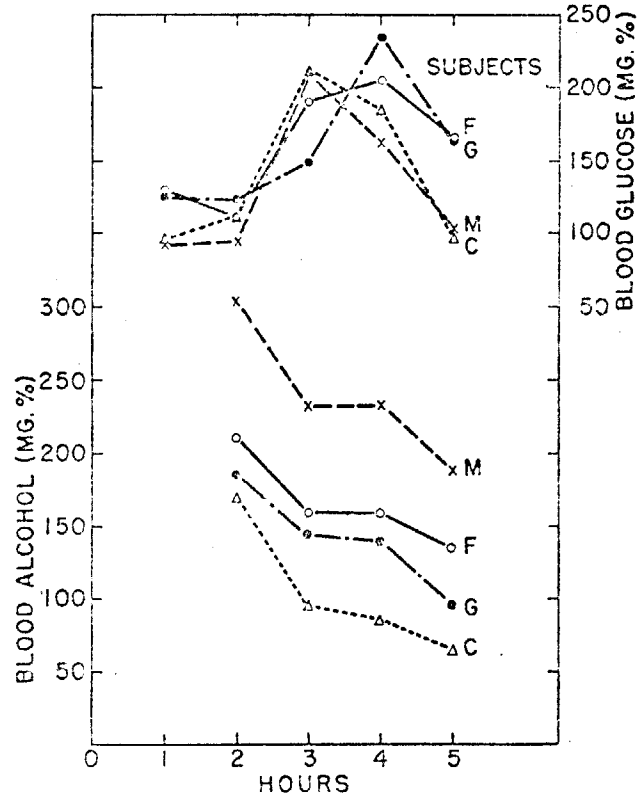

FIG. 1. The effect of sucrose administration on blood glucose and alcohol disappearance. Subjects F, G, and C were given whisky to provide 1 gm. of alcohol per kilo of body weight, while Subject M was given enough to provide 1.5 gm. per kilo. 50 gm. of sucrose were given to all subjects at 2 hours.

All subjects studied showed mild clinical effects of alcohol intoxication. Their mood became elevated; they talked freely and exchanged jokes. Shortly after sucrose ingestion, however, there was a striking change. The patients became quiet; all appeared drowsy and some dozed. A number of them complained of "sick stomach" and nausea. One patient who vomited was excluded from the study. Patients given alcohol without sugar had no manifestations of the latter signs and symptoms.

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DISCUSSION

In so far as is now known alcohol is oxidized by alcohol dehydrogenase to acetaldehyde and DPN (diphosphopyridine nucleotide) is a necessary coenzyme for this reaction. The acetaldehyde is rapidly oxidized, presumably to acetate, and this is further oxidized by entry into the tricarboxylic acid cycle. Pyruvate, acetate, and alcohol thus enter the common cycle at this stage and are probably indistinguishable metabolically. When the inhibition of $C^{14}O_2$ production from radioalcohol by pyruvate and acetate was first observed, we believed that this might be explained by a dilution effect at the acetate stage. Further studies showed, however, that alcohol oxidation was delayed at the first step, alcohol → acetaldehyde, since there was a reduced rate of alcohol disappearance from the blood (Table IV, Fig. 1). This effect could not be explained by dilution at the acetate level. It also seemed unlikely that there would be a shift in the equilibrium, acetate ⇌ acetaldehyde ⇌ alcohol, toward the alcohol, since this would presumably require the accumulation of high levels of acetaldehyde.

We suggest that the effect of pyruvate, acetate, and glucose represents a competition for DPN, since this coenzyme is also required for oxidation in the tricarboxylic acid cycle. When excess acetate is provided, the available DPN is preferentially reduced by the steps in the cycle which require it and relatively less is available for reduction by alcohol with alcohol dehydrogenase.

The results obtained after the administration of excess niacin support this conclusion. Although studies on the DPN and TPN (triphosphopyridine nucleotide) levels in tissues after the administration of high levels of niacin are not yet available, it is possible that an increase in the synthesis of the coenzymes was obtained. No significant inhibition of alcohol oxidation by pyruvate or acetate was observed in these animals. The results obtained with the thiamine-deficient animals also support this conclusion. Alcohol oxidation was normal in these animals and pyruvate had a smaller effect. Thus pyruvate *per se* does not inhibit alcohol oxidation directly.

It will be recalled that a requirement for niacin by the rat cannot be demonstrated by growth studies unless the level of tryptophan in the diet is very low. Under most conditions niacin may be considered as unessential for the rat consuming a diet containing 20 per cent of casein. Yet the diet used in these studies containing 30 mg. of niacin per kilo is apparently limiting for niacin under the conditions imposed by large loads of acetate or pyruvate and alcohol. The studies thus emphasize the necessity of attention to the diet in all types of metabolic studies. They also emphasize the complexity of nutritional requirements unless one defines the requirement to be met.

SUMMARY

In normal rats the administration of pyruvate or acetate inhibits the oxidation of alcohol as measured by the excretion of $C^{14}O_2$ after the administration of ethanol-1-C^{14}. Since the disappearance of alcohol from the blood is also delayed, the inhibition is apparently at the first stage of alcohol oxidation to acetaldehyde.

The administration of large amounts of niacin prior to study prevents the inhibitory effect of pyruvate. In thiamine deficiency the pyruvate inhibition is decreased. It is suggested that the inhibition is due to competition for the coenzyme DPN, this coenzyme being preferentially reduced in the tricarboxylic acid cycle rather than by alcohol oxidation when it is available in limited amounts.

Limited studies upon alcohol disappearance from the blood of human subjects suggest a similar effect.

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DRUG RESPONSES IN GANGLIA PERFUSED WITH SODIUM ACETATE

BY

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Depolarization of mammalian sympathetic ganglia by acetylcholine and other stimulating drugs depends upon the presence in the extracellular fluid of inorganic cations. Although sodium is the major cation involved in drug-induced depolarization, calcium can serve as an effective mediator of the response under appropriate conditions. By contrast, lithium has been found to be relatively ineffective as replacement for sodium in mammalian ganglia (1, 2).

In the present study, an attempt was made to determine the role of chloride, the primary extracellular anion, in ganglionic responses to drugs. This was done by perfusing the superior cervical ganglia of cats with media containing acetate as the dominant anion. Since the permeability of excitable tissues to acetate is considerably less than that to chloride (3), the effect of replacing chloride with acetate is to create an anion-deficient environment for the ganglion cells.

MATERIALS AND METHODS

Surgical Procedures. The 14 cats used in this study were anesthetized with a mixture of sodium diallybarbiturate (70 mg/kg), urethane (280 mg/kg) and monoethylurea (280 mg/kg) administered intraperitoneally.

After ensuring patency of the airways by intubation of the trachea, the superior cervical ganglia were readily exposed following eversion of the upper portions of the trachea and the esophagus into the animal's mouth. The left superior cervical ganglion and a postganglionic (external carotid) branch were prepared for recording by removal of surrounding connective tissue. A cotton ligature soaked in Locke's solution was used to crush the postganglionic nerve at its point of contact with the external carotid artery. A deep cervical well was formed by tying skin flaps to the metal framework. The exposed area was covered with mineral oil.

In order to limit the flow of perfusion fluid primarily to the ganglion, all the major branches of the common carotid artery excepting those supplying the ganglion were tied and divided. The external carotid artery was ligated above the occipital arteries. The lymph gland lying lateral to the common carotid artery and below the carotid bifurcation was excised. Isolation of the venous side of the ganglion's vasculature and collection of perfusion fluid were not performed.

Perfusion of the ganglion was accomplished with a constant infusion pump which was set to operate at a rate of 0.4 ml/min. The solutions were introduced into the vasculature of the ganglion by means of a perfusion needle placed in the common carotid artery at a point just above the sternum. Blood flow to the ganglia was prevented by ligating the common carotid artery just below the entrance of the perfusion needle. To ensure that no blood mixed with perfusion fluid through collateral channels, the animal was killed by bleeding after the perfusion flow had been established.

All experiments were carried out at room temperature. The range of temperatures recorded during the experiments from the cervical well was 24° to 28° C. Drug injections were made through a 27-gauge needle secured in a holder and clamped to the framework. The injection needle was located just anterior to the perfusion needle to facilitate transport of the drug solutions toward the ganglion. The volume of injected solutions never exceeded 0.2 ml and the drugs were dissolved in the perfusion medium.

Perfusion Fluids. The standard sodium-Locke's solution used for perfusion was a bicarbonate-buffered saline medium having the following composition: 154 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 6 mM $NaHCO_3$, and 5.5 mM glucose. The acetate-Locke's solution used for perfusion contained: 160 mM Na acetate, 5.6 mM $KHCO_3$, 2.2 mM $CaCl_2$, and 5.5 mM glucose.

All the solutions were gassed with a mixture of 96.5 % O_2-3.5 % CO_2 and passed under pressure through a fritted glass filter of medium porosity. The media were placed in separatory funnels and connected to the infusion pump by a column of mineral oil. Displacement of the mineral oil from the pump forced the perfusion fluid into the ganglion at a rate of 0.4 ml/min.

Recording Procedure. The preganglionic nerve was freed from the carotid sheath, decentralized and suspended on a glass hook in the mineral oil. Platinum electrodes were placed on this nerve for stimulation purposes. Supramaximal stimuli of 0.1 msec duration were delivered at several frequencies (see Results); the stimuli were isolated electronically from ground.

Bipolar silver-silver chloride electrodes were positioned on the surface of the ganglion and the crushed end of the postganglionic nerve for recording changes in the ganglion injury potential. The electrodes were connected to a cathode-follower circuit that was, in turn, coupled to a resistance-coupled preamplifier. The amplified potentials were displayed on one beam of a dual beam oscilloscope. In all the records, an upward deflection of the tracing indicates a negativity of the ganglion with respect to the crushed end of the postganglionic nerve. Ganglion negativity and positivity are referred to as depolarization and hyperpolarization, respectively. Permanent records were made with a kymographic camera whose speed determined the time base of the system.

The doses of acetylcholine chloride, hexamethonium chloride, dimethylphenylpiperazinium iodide, and potassium acetate are expressed in terms of salts and refer to intra-arterial injections.

RESULTS

Effects on transmission. Striking changes in the contour of the ganglionic action potential were noted when the sodium chloride

(154 mM) of the perfusion fluid was replaced by sodium acetate (160 mM). As shown by the records of Figure 1, the changes occurred in two phases and affected both the spike and the afterpotentials. During the first 10-min period of perfusion with the acetate-containing medium, there was an increase in spike amplitude (30 to 300 % with a mean per cent-increase of 94 % in 6 experiments), a decrease in the positive potential, an increase in the negative afterpotential, and no change in the positive afterpotential. After that time, the increase in spike amplitude was maintained but the contour of the afterpotentials was modified a second time. With continued perfusion of the ganglion with the acetate solution, the afterpotentials were either markedly reduced (FIG. 1) or

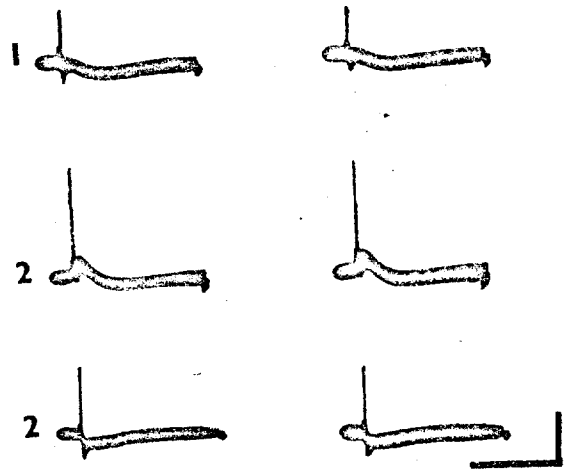

FIG. 1

Action potentials recorded from the surface of the superior cervical ganglion during perfusion with Locke's solution (1) and with a medium containing sodium acetate (2) as a replacement for sodium chloride. The ganglion was stimulated at a rate of 0.2 cps.

The top pair of records (1) show the responses evoked during perfusion with Locke's solution. The middle pair of action potentials (2) were obtained 5 min after perfusion with acetate-Locke's solution had started. The bottom responses (2) were obtained 15 min after perfusion with the acetate-containing solution.

The vertical calibration is 500 μV for the top and middle pair of records and 1 mV for the bottom pair of records. The horizontal calibration is 300 msec.

abolished (see FIG. 4, bottom row). Removal of the sodium acetate and restoration of the sodium chloride to the perfusion fluid resulted in a gradual (40 to 60 min) reversal of the above changes in the ganglionic action potential.

Stimulation at a rate of 2.0 cps for 3 to 5 min of the ganglia perfused

with Locke's solution resulted in a gradual decline of spike amplitude to 60 to 80 per cent of control values (response to single stimulus). By contrast, in two ganglia perfused with acetate-Locke's solution, stimulation at a rate of 2.0 cps for 1 min caused almost complete (greater than 95 %) abolition of the spike. In two additional experiments, it was observed that the hyperpolarization occurring during stimulation at a rate of 2.0 cps for 10 to 20 sec failed to occur in ganglia that had been perfused with acetate-Locke's solution for more than 10 min.

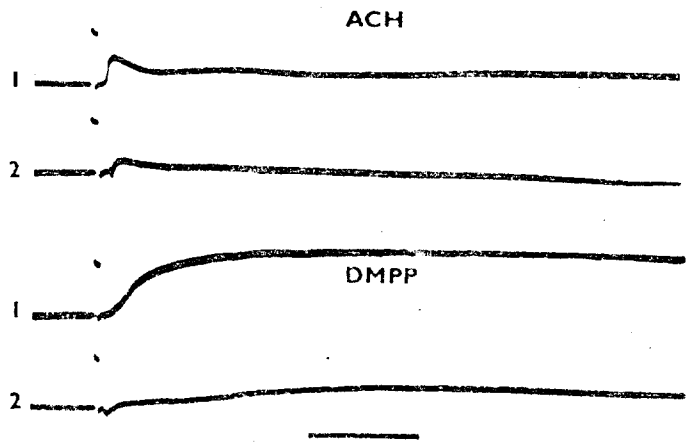

FIG. 2

Ganglionic surface potentials produced by acetylcholine (ACh) and dimethylphenylpiperazinium (DMPP) during perfusion with Locke's solution (1) and acetate-Locke's solution (2).

The monophasic waves of depolarization evoked by 20 μg of ACh are shown on the top pair of tracings; the responses to 2.5 μg of DMPP are shown on the bottom pair of tracings. The vertical and horizontal calibrations are 500 μV and 4 sec, respectively.

Effects on drug-induced depolarization. Acetylcholine (20 to 40 μg), dimethylphenylpiperazinium (2.5 to 10 μg) and potassium acetate (1 to 2 mg) caused a monophasic wave of depolarization when added to the perfusion stream of ganglia perfused with sodium-Locke's solution (FIGS. 2 and 3). However, in ganglia perfused for 10 to 15 min with acetate-Locke's solution, depolarization by acetylcholine (6 trials in 3 experiments) and dimethylphenylpiperazinium (8 trials in 4 experiments) was depressed severely (FIG. 2). Restoration of the response to control values occurred 30 to 60 min after reinstitution of perfusion with sodium-Locke's solution.

In contrast to that produced by the cholinomimetic drugs, the de-

polarization evoked by potassium acetate (2 trials in 2 experiments) was augmented considerably in ganglia perfused with acetate-Locke's solution (FIG. 3).

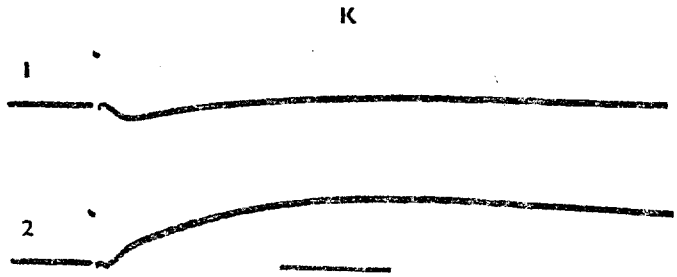

FIG. 3

Depolarization by potassium acetate (2 mg; K) in ganglion perfused with Locke's solution (1) or acetate-Locke's solution (2).

The vertical and horizontal calibrations are 500 μV and .4 sec respectively.

Ganglionic blockade in ganglia perfused with sodium acetate. In ganglia stimulated at a rate of 0.2 cps by supramaximal preganglionic volleys, hexamethonium (20 and 40 μg, 2 experiments) caused the same degree of blockade (60 and 80 % decrease in spike size) in ganglia perfused with acetate-Locke's solution as in ganglia perfused with sodium-Locke's solution. On the other hand, ganglion blockade by dimethyl-

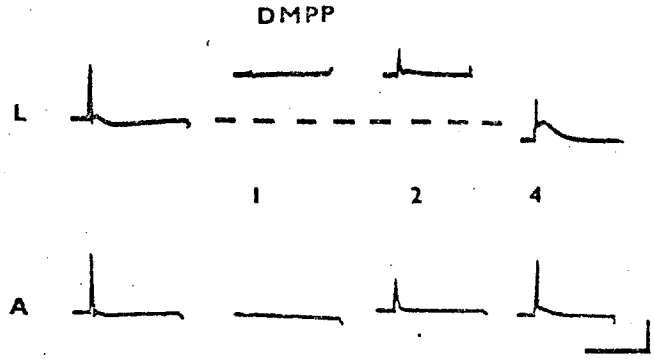

FIG. 4

Ganglion blockade by dimethylphenylpiperazinium (DMPP, 2.5 μg) in ganglion perfused with Locke's solution (L) and with acetate-Locke's solution (A).

In the top row of records, the dashed line marks the preinjection reference line. Deflections above the line indicate depolarization. The reponses evoked 1, 2, and 4 min after the injection of DMPP are shown. The vertical and horizontal calibrations are 500 μV and 300 msec, respectively.

phenylpiperazinium (FIG. 4) was modified in ganglia perfused with acetate-containing solutions.

Nicotinic cholinomimetic agents cause ganglionic blockade in two discrete phases, during depolarization and during hyperpolarization (4, 5, 6). As noted above, ganglionic depolarization by dimethylphenylpiperazinium ions was depressed in ganglia perfused with acetate-Locke's solution. Nonetheless, ganglionic blockade that coincided with depolarization was unaffected (5 experiments). Like depolarization, the hyperpolarization produced by dimethylphenylpiperazinium 3 to 5 min after injection of the drug was abolished in acetate-perfused ganglia. Similarly, the late-occurring blockade of the ganglia was diminished in ganglia perfused with acetate-Locke's solution (FIG. 4).

DISCUSSION

The changes in mammalian sympathetic ganglia resulting from the replacement of external chloride by the less permeant acetate ion can be explained best in terms of ganglionic cellular depolarization. In those cells where chloride contributes to membrane conductance, removal of external chloride leads to an unbalanced leakage of internal chloride and a reduction in the membrane potential (3). Although direct evidence of chloride efflux remains to be determined in sympathetic ganglia, it is reasonable to propose that the loss of chloride by the ganglion cells during perfusion with acetate-Locke's solution was the basis of the observations described above.

Evidence that the ganglia were depolarized when acetate replaced chloride in the perfusion fluid is indicated by the following. First, abolition of the afterpotentials and an increase in spike amplitude are characteristic of depolarized sympathetic ganglia (7, 8). Second, the decrease in amplitude of depolarization by nicotinic drugs accords with persistent depolarization and the nonregenerative nature of drug-evoked depolarization (9). A variety of procedures leading to ganglionic depolarization diminish the depolarization produced by cholinomimetic drugs. Finally, the elimination of ganglionic hyperpolarization and blockade caused by dimethylphenylpiperazinium in acetate-perfused ganglia are predictable consequences of depolarization. It has been shown in other studies (6) that depolarization by anticholinesterase agents, pilocarpine or tetanic stimulation prevented both responses to nicotinic drugs.

Note should be made also of the fact that the increase in negative afterpotential observed during the first minutes of perfusion with ace-

tate-Locke's solution has been described in other tissues deprived of external chloride ions (3). However, it is clear that the early change in ganglionic afterpotentials cannot be attributed to depolarization of the ganglion cells. Like drug-induced depolarization, the negative afterpotential is decreased by depolarizing procedures and enhanced by hyperpolarizing procedures.

Finally, it should be noted that frog sympathetic ganglia apparently differ from mammalian ganglia insofar as chloride ions are concerned. It has been reported (10) that the substitution of methyl sulfate for chloride in the medium bathing frog ganglion cells had no effect on the membrane potential. Frog sympathetic ganglia, unlike their mammalian counterparts, also appear to lack muscarinic receptors (11).

Summary

Substitution of sodium acetate for sodium chloride in the media perfusing cat superior cervical ganglia resulted in a number of alterations in responses to nerve stimulation and ganglionic drugs. During the first 10-min of perfusion with acetate-containing media, there was an increase in spike amplitude and the negative afterpotential. After that time, all afterpotentials were reduced or abolished. Similarly, depolarization by nicotinic drugs was reduced. Ganglionic hyperpolarization and blockade by a nicotinic drug was eliminated. The late-occurring changes produced by the substitution of acetate for chloride are explained in terms of ganglionic depolarization due to the loss of internal cellular chloride.

Acknowledgment

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Volatile Compounds from Heated Glucose

SUMMARY—Volatiles from anhydrous glucose heated in air at 250°C for 30 min were collected in a trap maintained at the temperature of solid carbon dioxide. A concentrated ether extract of the distillate was shown by gas chromatography to contain at least 100 compounds. Among those identified and heretofore unreported from heated glucose were the gamma lactone of 4-hydroxy-2-pentanoic acid, 1-(2'-furyl)-propane-1,2-dione (acetylfuroyl), 3-methylcyclopentane-1,2-dione, phenol and methylfuroic acid.

INTRODUCTION

A NUMBER OF PUBLICATIONS have recently appeared on the application of gas chromatography to the analysis of volatile products from the thermal decomposition of carbohydrates and closely related substances: Bailey *et al.* (1962), Bryce *et al.* (1963), Doerr *et al.* (1966), Fiddler *et al.* (1966), Gianturco *et al.* (1963, 1964), Greenwood *et al.* (1961), Graham (1966), Heyns *et al.* (1966a), Sugisawa (1966), and Tai *et al.* (1964). These studies indicate the presence of a wide variety of compounds, but generally only the low boiling classes have received major attention. Compounds of intermediate volatility have not been as extensively studied despite some indication that they may be more important contributors to flavor.

Since glucose was present in substantial amounts in the materials previously studied, it was felt that heating glucose itself in an isolated system would give valuable information on the origin of the volatile flavor compounds.

Among the variables that may affect the composition of volatiles from glucose are heating time, temperature, and the presence of oxygen. On the basis of achieving an adequate collection of volatiles in a reasonable time, a temperature roughly intermediate between 150°C (Sugisawa, 1966) and 300°C (Heyns *et al.*, 1966a) and a heating period of 30 min were chosen. The heating was carried out in atmospheres of both air and nitrogen.

EXPERIMENTAL

Heating and distillation

Two g of anhydrous glucose (Baker analyzed reagent) were placed in a 50 ml (₩ 45/50) three-neck distillation flask in which a coarse capillary and a thermometer were fixed by means of Teflon adapters (Fig. 1). A side-arm of the flask led into a trap which was surrounded by dry crushed ice. The system was connected to a water aspirator or to a cylinder of "pre-purified grade" N, to obtain either the air or N atmosphere over the heated glucose. Heat was provided by an electric heating mantle which was thermostatted by a Jelrus automatic controller (manufactured by the Jelrus Co., Inc., New York, N. Y.) to maintain an internal flask temperature of approximately 250°C. The total distillation time was 30 min.

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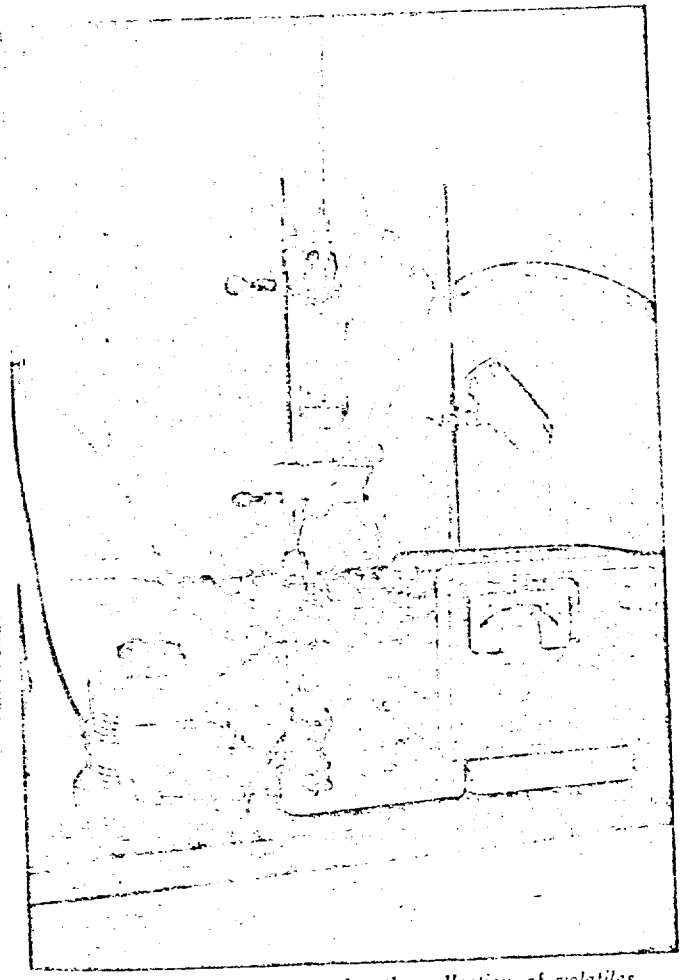

Fig. 1. Distillation system for the collection of volatiles.

Extraction and concentration

The frozen distillate, usually less than 1 ml, was diluted to about 3 ml with distilled water, transferred to a 150 × 16 mm test tube, enough anhydrous Na_2SO_4 added to saturate the solution, and extracted with an equal volume of redistilled, anhydrous ether. The ether phase was then pipetted into a 5 ml beaker, and the ether slowly evaporated under a stream of N to about one-third the original volume, after which, it was transferred with a pipet to a 3 ml conical bottom, glass-stoppered centrifuge tube for further evaporation under N. The final concentration was arbitrary, but evaporation was terminated when there was a noticeable increase in viscosity of the ether extract. The final volume was about 0.5 ml. This concentrate had a refrigerated storage life of 7–10 days at 3°C, after which, it tended to form a precipitate. Stability could be increased somewhat by redilution with anhydrous ether prior to storage.

When it was desired to eliminate the masking effect of the ether solvent on compounds of very short retention times during subsequent gas chromatography, the undiluted distillate was transferred directly to the centrifuge tube and diluted with 2.0–2.5 ml of a saturated Na_2SO_4 solution, then centrifuged at 4000 rpm until an oily, brown ring separated at the surface. From this layer, 0.5 μl samples were withdrawn for direct injection into the gas chromatograph-mass spectrometer unit.

Analysis

One to five micro-liter samples of the concentrated ether extract were taken for gas chromatography. Mass spectra were obtained, using a coupled gas chromatograph (Perkin-Elmer Model 226)—mass spectrometer (Hitachi-Perkin-Elmer Model RMU-6A) unit with a capillary column, 200 ft long, 0.02 in. ID, and coated with a mixture of diethyleneglycolsuccinate and H_3PO_4 (2%). The column effluent was split with equal portions being directed to the flame ionization detector, and to the mass spectrometer, the latter via a Watson-iBemann, helium separator (Watson *et al.*, 1965). The operating parameters were as follows:

Gas chromatograph:	
flow rate	Helium carrier gas, at 15 ml/min.
program	30°C for 5 min, then 2°C/min to 160°C
injection block temperature	275°C
Mass spectrometer:	
inlet temperature	200°C
ion source pressure	4×10^{-6} mm
ion source temperature	250°C
acceleration voltage	2.5 Kv
target current	60uA
electron multiplier voltage	2.5 Kv
scan speed	12 sec. (m/e 12-400)
exit slit	0.15 mm

Trapping for infrared analysis was attempted by the method of Edwards *et al.* (1965), from a Perkin-Elmer 810 gas chromatograph with a 6 ft long, ⅛-in. OD column packed with 10% polypropyleneglycol on 60/80-mesh Chromosorb W. The outlet temperature was kept at 250°C, and the injection block temperature at 275°C. The column was held at 65°C for 5 min, and then ballistically programmed to 200°C where it remained until completion of the analysis. Twenty % of the carrier gas, N was diverted to the flame ionization detector, and 80% to the outlet.

The presence of formaldehyde and CO_2 was established in separate experiments by bubbling the sweep gas from the heated glucose into a solution of 1% chromotropic acid in concentrated H_2SO_4 (Frisell *et al.*, 1958) and 0.05% $Ba(OH)_2$ solution respectively. The development of a purple color in the former and a precipitate in the latter was taken as evidence of the presence of these compounds.

RESULTS AND DISCUSSION

On the basis of comparative gas chromatography, there was no evidence that the heating atmosphere significantly influenced the composition of the collected volatiles.

Since primary emphasis in this study was placed on compounds of intermediate volatility, no special attempt

Table 1. Compounds identified in the volatile fraction of heated glucose.

G.C. No.	Compounds	Method of identification I.R.	G.C.	M.S.	Remarks
	CO_2				See experimental
	Formaldehyde				See experimental
1	Furan		++	++	
2	2-Methylfuran		++	++	
2	Butadione (Diacetyl)		++	++	
4	Acetic acid		++	++	
6	2-n-Propylfuran			+	Heyns *et al.* (1966b)
8	Furfural (2-furaldehyde)	++	++	++	
9	2-Acetylfuran		++	++	
12	3-Furaldehyde			+	Heyns *et al.* (1966b)
15	5-Methyl-2-furaldehyde		++	++	
16	5-Methyl-2-acetylfuran			+	
24	4-Hydroxy-2-pentenoic acid, gamma lactone		++	++	
27	1-(2'-furyl)-propane-1,2-dione (acetylfuroyl)		++	++	
29	3-Methylcyclopentane-1,2-dione		++	++	
31	Phenol		++	++	
42	Methylfuroic acid (one of various positional isomers.)				By mass spectrum interpretation only

++ Agreement with authentic compounds.
+ Mass spectral data only.

was made to conserve the more volatile components during distillation. Many of these have recently been reported by Qua *et al.* (1964), Heyns *et al.* (1966a), and Sugisawa (1966).

At least 100 compounds were recovered by the method of distillation and concentration employed, as shown by a typical gas chromatogram (Fig. 2). Table 1 lists the compounds identified and their method of identification.

Identifications were made by first obtaining mass spectra of the eluting peaks to ascertain probable structures which were subsequently confirmed or rejected on the basis of comparison with mass spectra of authentic samples. Confirmation of a specific structure was then determined by comparison of gas chromatographic retention times with authentic samples.

Except for furfural, attempts to obtain useful infrared spectra from the packed column were unsuccessful, possibly because of the low concentrations involved, and the low efficiency of recovery from the injected sample.

Of the compounds identified, the gamma lactone of 4-hydroxy-2-pentenoic acid 1-(2'-furyl)-propane-1,2-dione ,acetylfuroyl), 3-methylcyclopentane-1,2-dione, phenol and methylfuroic acid are not heretofore known to have been reported as arising from heated glucose. The mass spectra of these compounds are presented in Fig. 3.

The existence of phenol as a volatile compound from heated glucose is observed with special interest, due to its known toxic properties. The presence of the gamma lactone of 4-hydroxy-2-pentenoic acid is noteworthy, because of the implication of unsaturated gamma lactones in anti-

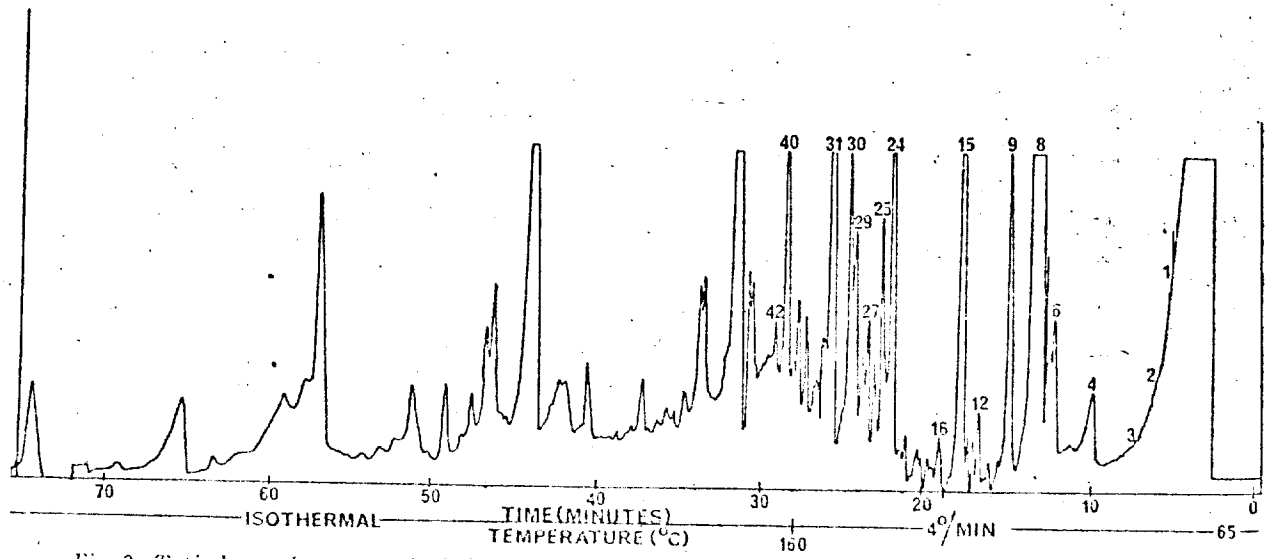

Fig. 2. Typical gas chromatogram of the volatiles obtained from the distillation of glucose at 250°C for 30 min.

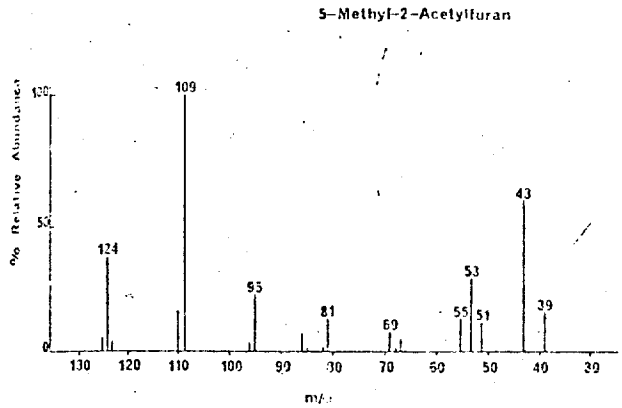

Fig. 3. Mass spectra of some compounds of intermediate volatility identified in the distillate of glucose heated at 250°C for 30 min.

biosis (Oxford 1945). Also, an enolic isomer of 3-methylcyclopentane-1,2-dione is reported as being used to impart a maple flavor and as a flavor potentiator by Filipic *et al.* (1965).

The mechanism of formation of 4-, 5-, and 6-carbon furan structures from monosaccharides has already been proposed Newth (1951), Anet (1964), and Byrne *et al.* (1966), but the presence of 7-carbon furan compounds in the volatiles from heated glucose (propylfuran, methylacelytfuran and 1-(2′-furyl)-propane-1,2-dione leads to the suspicion that secondary reactions of primary decomposition products also influence the vapor phase composition. For example, acetic acid and furfural could conceivably react to form 1-(2′-furyl)-propane-1,2-dione. In previous works, this compound was obtained only as an oxidative product of 2,furylacetone (Cosgrove *et al.*, 1952) and one component of coffee volatiles (Gianturco *et al.*, 1964). Such a compound, however, could also originate from a polymer fragmentation process.

A common source of benzenoid and alicyclic compounds is the pyrolysis of carbonaceous matter (bituminous coal) at very high temperatures (1000–3000°C), in the absence of air. The presence of phenol and 3-methylcyclopentane-1, 2-dione at 250°C may therefore indicate a common reaction path, but the details of the mechanism of formation of these structures from heated glucose has yet to be elucidated.

Most of the compounds identified have a unique, mild aroma none of which could be described as that of caramel. It may therefore be that the caramel aroma itself arises from the mixture of volatiles present.

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Effect of Sex, Feathering, Rate of Growth and Acetates on the Chick's Need for Glycine

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NON-DIETARY factors that have been reported or suggested as influencing the glycine requirement of the growing chick are rate of feathering (by Hegsted *et al.*, 1941), the low level of [illegible] in muscle creatine and age of chick by [illegible] (1951) and sex of chick by Douglas *et al.* (1958). In addition, Almquist and Mecchi (1940) reported a growth response when their casein-brewers yeast-fish meal diet was supplemented with either sodium acetate, ammonium acetate or glycine.

In view of the above reports and the findings of Waterhouse and Scott (1961a, b) that both male and female chicks fed a casein diet require a high level of glycine for optimum growth, the following experiments were designed to determine whether some of the factors that have been reported as exerting an influence on the chick's need for glycine could be substantiated.

EXPERIMENTAL

Newly hatched chicks from the mating of New Hampshire males to Columbian females were fed a practical diet composed largely of corn and soybean oil meal for a pretest period of 7 days. On the 7th day [illegible]

and water were furnished on an *ad libitum* basis. Feed consumption and weights were recorded at 14 days of age and at the termination of each experiment. Each diet was fed to duplicate groups of 10 chicks each in all experiments for the period 7-20 or 7-21 days of age.

The casein basal ration used in the following experiments is given in Table 1. The diet contained 20 percent protein which is recognized to be suboptimal for this diet (Scott *et al.*, 1957; Waterhouse and Scott, 1961b). The basal diet was supplemented with [illegible] percent arginine HCl and 0.3 percent DL-methionine. By calculation, the diet contained 1.7 percent arginine, [illegible] percent total sulfur amino acids and 0.4 percent glycine. This is the same basal diet Waterhouse and Scott (1961a) used to demonstrate that chicks fed 20 percent protein from casein require

TABLE 1.—Composition of casein [illegible]

	Percent
[illegible]	[illegible]

TABLE 2. *Effect of sex and of rate of feathering on glycine needs of chicks in this test*

Supplemental glycine added to basal diet[1]	Sex	Av. gain (grams)[2]	Increase in gain over basal (%)	Gain/Feed
None	M	165		.55
0.5%	M	174	5.4	.58
1.0%	M	186	12.7	.60
2.0%	M	187	13.3	.60
3.0%	M	203	23.0	.68
None	F	164		.56
0.5%	F	169	3.0	.58
1.0%	F	180	9.7	.60
2.0%	F	186	13.4	.63
3.0%	F	194	18.3	.67

[1] As described in Table 1.

[2] Average of triplicate groups of 10 male or 10 female chicks each per treatment with an average initial weight of 67 grams.

from 2 to 4 percent supplemental glycine for maximal growth and feed utilization.

RESULTS AND DISCUSSION

Experiment 1. The presence of the sex-linked rapid-feathering gene (K) in the crossbred female chicks and the slow-feathering gene (k) in the crossbred male chicks used in our laboratory (New Hampshire ♂ × Columbian ♀) offered an opportunity to determine whether the sex of chicks with different feathering rates influences the chick's need for glycine. The design and results of this test are recorded in Table 2. Increments of glycine were added to the cavin basal diet and fed to both male and female chicks having identical average weights at the start of the experiment. The growth response of both male and female chicks at equivalent levels of glycine was quite similar. Analysis of variance showed that only the effect of glycine was significant and neither sex nor the interaction of glycine with sex was significant.

Experiment 2. From a population of approximately 3600 male chicks raised to 7 days of age on a corn-soybean ration, 9 groups of 150 chicks each were selected on the basis of their ability to grow during the 7-day pretest period. The group designated as "fast-growing" averaged 78 grams while the second group, "slow-growing," averaged 57 grams. Growth during the assay period has been recorded as (1) gain in weight and (2) as instantaneous percentage rate of growth (Brody, 1945). The response to supplemental levels of glycine is indicated in Table 3. Groups of chicks initially classified as "fast-growing" continue to gain more in absolute weight than the "slow-growing" groups at each of the levels of glycine. However, chicks initially classified as "slow-growing," in the manner described gained more weight on a percentage basis than did those classified as "fast-growing," a situation which has been previously noted by Grimminger and Scott (1959). The response to increments of glycine was maximal at 2 percent supplemental glycine in the case of the "fast-growing" chicks (Table 3). An analysis of variance of either the absolute gain in weight or instantaneous rate of growth is

TABLE 3. *The glycine need of the chicks in relation to growth potential*

Supplemental glycine added[1]	Av. gain (grams) 7–21 days[2]		Growth rate[3]		Gain/Feed	
	A	B	A	B	A	B
None	214	161	9.43	9.64	.61	.50
0.5%	215	173	9.44	9.92	.61	.52
1.0%	215	177	9.50	9.91	.62	.57
1.5%	219	178	9.53	9.98	.61	.56
2.0%	237	183	9.96	10.25	.68	.62
2.5%	232	188	9.67	10.39	.68	.63

[1] Basal diet as described in Table 1 with 1.50 percent total glycine (1.1 percent supplemental).

[2] Average of triplicate groups of 10 male chicks each per treatment.

[3] Instantaneous percentage rate of growth parallel to $\frac{W_2 - W_1}{t_2 - t_1} \times 100$ (Brody, 1945)

where W_2 and W_1 represent chick's weights at t_2 and t_1 times, respectively.

A chicks classified as "fast-growing" and averaging 78 grams at 7 days when placed on test.

B chicks classified as "slow-growing" and averaging 57 grams at 7 days when placed on test.

cated that the effects attributable to glycine and weight gain were highly significant, but the interaction between glycine and weight gain was not significant. It is unlikely, therefore, that "genetic" differences in growth potential alters the chick's need for glycine when the requirement is expressed as a percentage of the diet.

Experiment 5. The purpose of this experiment was to investigate whether growth could be increased when the glycine deficient basal diet was supplemented with either sodium acetate or ammonium acetate. A quantity of sodium or ammonium acetate calculated to be equimolar to 3 percent glycine was added alone and in combination with 3 percent glycine to the casein basal diet. Within 48 hours after the initiation of this experiment, some mortality had occurred among chicks receiving the diets containing sodium acetate and many of the survivors showed symptoms of ataxia and anoxia. Gross post mortem examination showed hypertrophy of the kidneys and gizzards, with some deposits in these organs. A white precipitate was noted in the pericardium as well as the peritoneum. The lesions were not unlike those noted by Carroll (1941) when chicks were fed diets containing the sodium salts of either citric or acetic acid. Carroll reported that potassium citrate was capable of some less of alleviat-

TABLE 5. [illegible] *and weight gain of chicks fed casein diet supplemented levels of sodium acetate or citric acid*

Treatment	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible] *Table 5.*

ing the toxicity of the sodium salts. Supplementing the glycine deficient basal diet with ammonium acetate also depressed chick growth and to about the same degree as supplemental sodium acetate. However, the ammonium compound unlike sodium acetate did not exert an adverse effect on [illegible] (Table 5). Growth was enhanced when the ammonium acetate containing diet was supplemented with glycine whereas glycine was without effect when the chicks were fed with sodium acetate.

Experiment 6. Since chicks were unable to tolerate the level of sodium acetate used in Experiment 5, a second feeding trial was made to ascertain the growth promoting potential of this compound at lower levels and to determine the chick's requirement for [illegible] required to induce symptoms of toxicity. In Experiment 6, the cocks and pullets which are listed in Table 6, were grouped among [illegible]

[illegible]

[illegible] the female chicks receiving the sev[illegible] were [illegible] for rapid [illegible] [illegible] [illegible] of [illegible] [illegible] [illegible] [illegible] [illegible]. However, no evidence could be obtained which would suggest that wide differences in [illegible] diffs, [illegible] affect the chick's [illegible] [illegible].

Nature [illegible] [illegible] [illegible] increase of chick growth when added to a [illegible] deficient basal diet. Sufficient sodium acetate at a concentration of 2.0 percent or more was shown to be toxic for chicks as measured by survival rate.

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THE ACTIONS OF METABOLIC SUBSTRATES AND INHIBITORS ON THE RABBIT AURICLE*

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Knowledge of cardiac muscle metabolism has advanced during the past few years to an appreciable extent, but the correlation of metabolic processes with the various functions of the heart has been very little investigated. It is possible that some of the enzyme systems that are highly active in cardiac extracts or homogenates, or even in heart slices, are not intimately involved in the beating of the heart. It is also possible that in the intact beating heart there are enzyme systems of great importance, which are either destroyed or altered by any damage to the myocardium. Such systems may be brought to light by a more detailed analysis of the actions of metabolic substrates and inhibitors on the normally contracting cardiac tissue. For the understanding of drug action on the heart, it is frequently necessary to correlate the metabolism with the functional aspects of impulse discharge, conduction, and contraction. The isolated rabbit auricle was used in the present work because both the amplitude of contraction and the rate of impulse discharge could be more accurately measured with it than with the entire heart where there occur problems of conduction block and complex contractile movement. It is also difficult to maintain normal conditions in the perfused heart, and the tissue soon becomes damaged, whereas auricles will beat regularly for many hours in the proper medium. This work was done in connexion with the problem of the action of acetylcholine on the heart and of how this action is influenced by the metabolic state of the myocardium. The results obtained with acetylcholine will be reported in a subsequent paper.

METHODS

The effects of metabolically important substances on the rabbit auricle were determined by addition of the substances to a bath in which the auricles were beating. Hearts from young rabbits were used, inasmuch as it was found that such preparations beat more regularly and give more reproducible results than those from older rabbits. It is possible that in older animals the auricular walls are so thick that diffusion of oxygen into the tissue, or diffusion of metabolic products from the tissue, is not adequate. It also seemed that better results were obtained when the rabbits were allowed to remain quiet for several minutes before they were killed. Auricles obtained from animals that had been killed immediately after being caught and handled often gave poor results

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and would seldom achieve a regular beat. The rabbits were killed by a blow on the head and the hearts were rapidly removed. The hearts were allowed to beat for about half a minute in a dish of physiological medium in order to allow as much blood as possible to be pumped out of the tissues. The ventricles were often massaged in order to rinse the blood from the auricles, care being taken not to touch the auricles. The auricles were then cut from the heart and carefully trimmed so that no ventricular tissue remained. Caution was exercised in the trimming of the arteries and veins that no damage be done to the adjacent regions of the auricle, for it is in these regions that the pacemaker tissue usually lies. The auricles were suspended in 70 ml. of Ringer solution (0.137 M-NaCl, 0.0027 M-KCl, 0.0018 M-$CaCl_2$, 0.00011 M-$MgCl_2$, 0.0[illegible]2 M-$NaHCO_3$, 0.00042 M-NaH_2PO_4, and 0.0055 M-glucose) which, after saturation with the gas mixture, had a *p*H of 8.1. Substances to be tested were added in 0.7 ml. vol. at a concentration 100 times that required in the final medium. Although this medium is a good buffer, all solutions of these substances were brought to a *p*H close to [illegible] before their addition to the bath. All measurements were made at 30° C. It was found that better results could be obtained at this temperature than at 38° C. At a higher temperature it is likely that the rapid rate often limits the recording of the amplitude and this would introduce serious errors into the determinations of amplitude effects. The auricles also beat regularly over a much longer period of time at the temperature. The medium was continually saturated with a mixture of 5 per cent [illegible] and 95 per cent O_2. The substances used were obtained in as pure a form as possible. The sodium fluoroacetate was kindly supplied by Dr. Saunders, of Cambridge University.

In order to achieve quantitative results the auricular tracings were carefully measured, the amplitude being recorded in millimetres and the rate of beating in contractions per ten seconds. It was necessary to use this small time unit for measuring the rates, inasmuch as rapid rate changes could not have been accurately determined if longer intervals had been chosen. The effects produced by the addition of any substance were expressed in terms of the percentage change in the amplitude and rate. Such results might conceivably be expressed as absolute changes, percentage changes, or as final levels of activity to which the auricles were brought by the substances. For most purposes the percentage change is the most significant. In the first place, owing to the recording arrangement, the free amplitude of contraction is not measured; hence, if absolute changes were given, it would not be possible to compare one experiment with another. Furthermore, in considering the effects of substances on the auricular frequency we are usually interested in the changes in the rates of processes occurring within the pacemaker cells. It is likely that the rates of such processes are roughly proportional to the frequencies of beating. In metabolic studies the effect of an inhibitor on an enzymatic system is usually expressed in terms of percentage inhibition, and so here we might expect a more direct relationship to the underlying processes if we also express the frequency changes in similar terms. Justification for this method of expression, with regard to both rate and amplitude, was found in the quantitatively comparable results obtained from auricles beating at greatly different rates and amplitudes.

The auricles usually beat somewhat irregularly for 5–30 minutes after placing them in the bath, but eventually become quite constant with respect to both rate and amplitude. Experimental work was always initiated after the auricles had reached this steady state. Such auricles would beat for two to three days, the amplitude gradually decreasing during the second and third days. No auricles were considered to be normal after the first day, and all results reported were obtained on fresh auricles. The recorded amplitude was usually adjusted to between 40 and 100 mm. Normal auricles beat spontaneously at an average rate of 102 contractions per minute, although there was quite large variation between the auricles from different animals in this respect, the extremes recorded being 72 and 144. At 38° C. the frequency is approximately 50 per cent

greater than at 30° C., and thus the rate at body temperature would have been approximately 184 beats per minute, a figure not far distant from the normal *in vivo* rate of the rabbit heart.

RESULTS

Effect of metabolic substrates on normal auricles

It is obvious that if a substance is to alter the beating of the auricles by acting through metabolic mechanisms, its quantitative effect will depend on the metabolic rate of the myocardial cells. If a particular substrate or intermediate, which is of importance in the functional metabolism of the heart, is deficient, we might expect its addition to produce an effect. Whether this would be stimulation or depression cannot be predicted until the correlation between the various metabolic reactions and the functioning of the heart cells is better understood. On the other hand, if an enzyme system is saturated with respect to its substrate (that is, if the substrate is not limiting the rate of the reaction), there may be no response to the addition of the substrate, or there may perhaps occur a retardation of the enzyme systems that operate before the particular step in which the substrate is involved. Furthermore, the observed action of substrates on the auricular tissue may be independent of energy mechanisms, the primary effect being upon the cellular membrane potential or the contractile substance itself. Lastly, the possible actions of these substances on the acetylcholine equilibrium in the cardiac tissue must be borne in mind. Since there is a slow formation of acetylcholine in the auricles (either within the heart cells themselves or released from the post-ganglionic nerve endings), it is possible that a substance may stimulate or depress this formation, or alter the rate at which this endogenous acetylcholine is destroyed by modifying the activity of the cholinesterases, or antagonize the action of the acetylcholine on the contractile cells.

The results obtained on the action of substrates on normal auricles are summarized in Table I. Changes of less than 5 per cent are probably not significant unless derived from experiments upon several auricles. An inspection of this Table will show that stimulation of either rate or amplitude is very seldom seen with any of the substrates investigated. It is likely that the only really significant stimulation is that produced by citrate on the rate. These substances, in general, have no effect on the normal auricle until a certain concentration is reached and then they begin progressively to depress it. This is seen in the concentration-action curves in Fig. 1. On the whole, it is probable that the enzymatic systems involved in providing energy for the beating of the auricles are not limited by any of the substrates tested.

The amplitude is more readily depressed by these substances than the rate. On the average, for any specific concentration of substrate, the depression of the amplitude is two to three times as great as the depression of the rate. However, substrates vary in this respect and it will be seen that acetate, at lower concentrations depresses the rate as strongly as it does the amplitude. The maximum effect, in most instances, is reached from one to two minutes after the addition of the substrate to the bath. There is then, with the majority of substrates used, a partial or complete spontaneous recovery from this depression. This was observed with pyruvate, lactate, fumarate, malate, acetate, oxalacetate, propionate, and butyrate; it was not seen with citrate or caprylate, the depression produced by these being

TABLE I

THE EFFECTS OF METABOLIC SUBSTRATES ON NORMAL AURICLES

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Succinate	0.0001	1	0	0	1	1
		5	0	+2	1	1
	0.001	1	0	0	1	1
		5	0	+3	1	1
	0.005	0.5	0	7	1	1
		1	0	9	2	5
		2	0	12	2	4
	0.01	0.5	18	22	3	3
		1	13	26	4	4
		2	10	27	3	3
Fumarate	0.001	0.5	6	4	1	1
		1	6	-5	1	1
		2	3	0	1	1
	0.005	0.5	3	5	2	2
		1	-3	7	3	3
		2	0	6	2	2
	0.01	0.5	9	8	2	3
		1	5	11	3	4
		2	2	13	3	3
Malate ..	0.001	0.5	3	0	2	2
		1	3	0	2	2
		2	6	1	1	1
	0.005	0.5	2	10	3	6
		1	4	14		5
		2	0	18	3	3
	0.01	0.5	4	9	2	3
		1	4	-18	2	3
		2	4	-20	2	2
		5	0	15	2	2
		10	0	8	2	2
		15	0	5	1	1
	0.02	0.5	-	23	1	1
		1	14	32	1	1
		2	7	27	1	1
		5	0	22	1	1
		8	0	12	1	1
		15	0	8	1	1
Oxalacetate ..	0.002	0.5	0	2	1	1
		1	0	-2	2	2
		2	0	0	1	1
	0.01	0.5	0	9	1	1
		1	0	14	1	1
		2	0	13	1	1
		3	0	11	1	1

TABLE I *contd.*

Substrate	Molar concentration	Time (min)	% Change in rate	% Change in amplitude	Hearts	Tests
Pyruvate	0.0001	1	0	0	2	2
		2	0	2	2	2
	0.001	0.5	0	2	2	2
		1	0	5	2	2
		2	0	6	2	2
		5	0	0	2	2
		10	0	3	2	2
	0.005	0.5	1	3	5	10
		1	1	6	7	13
		2	1	11	5	10
		3	2	9	3	5
		5	2	8	4	6
		10	4	5	3	3
		15	0	3	2	2
	0.01	0.5	0	9	2	2
		1	0	12	4	4
		2	0	16	5	5
		5	0	7	2	2
		15	5	6	1	1
	0.02	0.5	0	8	1	1
		1	8	3	1	1
		2	0	23	1	1
Acetate	0.00002	1	0	0	1	1
		5	0	0	1	1
	0.0001	1	6	8	1	1
		2	0	4	1	1
	0.0005	0.5	9	4	2	3
		1	22	15	2	3
		2	17	10	2	3
	0.001	0.5	5	9	4	5
		1	19	21	3	4
		2	11	9	3	3
		5	3	2	2	3
	0.01	0.5	4	8	2	2
		1	6	23	3	4
		2	4	16	2	3
		5	0	7	2	2
		10	16	3	2	2
		15	16	2	2	2
	0.03	1	6	27	2	2
		2	4	22	2	2
		5	0	18	1	1
		10	16	11	1	1
		15	16	15	1	1

TABLE I—*contd.*

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Propionate	0.0005	0.5	0	1	2	2
		1	0	4	2	2
		2	0	3	2	2
		5	3	8	2	4
		10	7	3	2	3
	0.005	0.5	7	10	1	2
		1	5	7	1	2
		2	0	3	1	2
		5	5	1	1	2
	0.01	0.5	2	4	1	1
		1	2	6	1	1
		3	0	3	1	1
Butyrate	0.0005	1	0	1	1	1
		5	0	0	1	1
	0.001	1	0	10	1	2
		2	3	8	1	2
	0.005	1	0	16	1	2
		2	6	11	1	2
		5	3	8	1	2
	0.01	0.5	0	7	1	1
		2	0	6	1	1
α-Hydroxy-butyrate	0.001	0.5	0	4	1	1
		1	0	6	1	1
		3	0	10	1	1
	0.005	1	7	11	1	1
		3	0	13	1	1
Caprylate	0.00002	0.5	0	0	1	2
		1	0	0	1	2
		3	0	4	1	2
		5	0	9	1	1
	0.0001	0.5	0	0	1	1
		1	0	5	1	1
		3	6	16	1	1
		5	6	26	1	1
	0.0005	0.5	6	0	1	1
		1	12	11	1	1
		5	6	24	1	1
	0.001	1	7	12	1	1
		2	14	28	1	1
	0.01	1	22	33	1	1
		2	22	42	1	1

TABLE I *contd.*

Substrate	Molar concentration	Time (min.)	% Change in rate	% Change in ampl.	Hearts	Tests
Glutamate	0.001	1	0	0	1	1
		2	0	0	1	1
	0.005	1	0	0	1	1
		2	0	0	1	1
	0.01	1	0	3	2	3
		2	0	1	2	3
	0.03	1	0	7	1	1
		2	0	5	1	1
Lactate	0.001	1	0	2	1	1
		2	0	1	1	1
	0.005	1	0	1	2	2
		2	0	1	2	2
	0.01	1	0	6	2	2
		2	0	2	2	2
		5	0	0	2	2
	0.03	1	0	9	1	1
		2	0	3	1	1
		5	0	2	1	1
Citrate	0.0001	1	0	0	1	1
		2	0	0	1	1
	0.001	0.5	0	5	2	3
		1	2	11	2	3
		2	2	12	2	3
	0.005	0.5	0	28	2	3
		1	3	47	2	3
		2	6	60	2	3
		4	2	62	2	3
	0.01	0.5	0	35	2	2
		1	16	63	2	2
		2	25	74	2	2
		5	28	75	2	2
		10	25	74	2	2
		15	20	72	2	2

irreversible until the substance was washed from the bath. Typical curves illustrating this spontaneous recovery are given in Fig. 2. This recovery is not due to destruction of the substrate, for in most instances the total amount of substrate present in the bath is sufficient to prevent any appreciable decrease in the concentration resulting from amounts destroyed by the tissue in the short time intervals involved. This type of spontaneous recovery is seen in cardiac tissue under the influence of a variety of substances, and is probably to be interpreted in the light of the ability of the cardiac functional systems to adjust themselves to abnormal conditions.

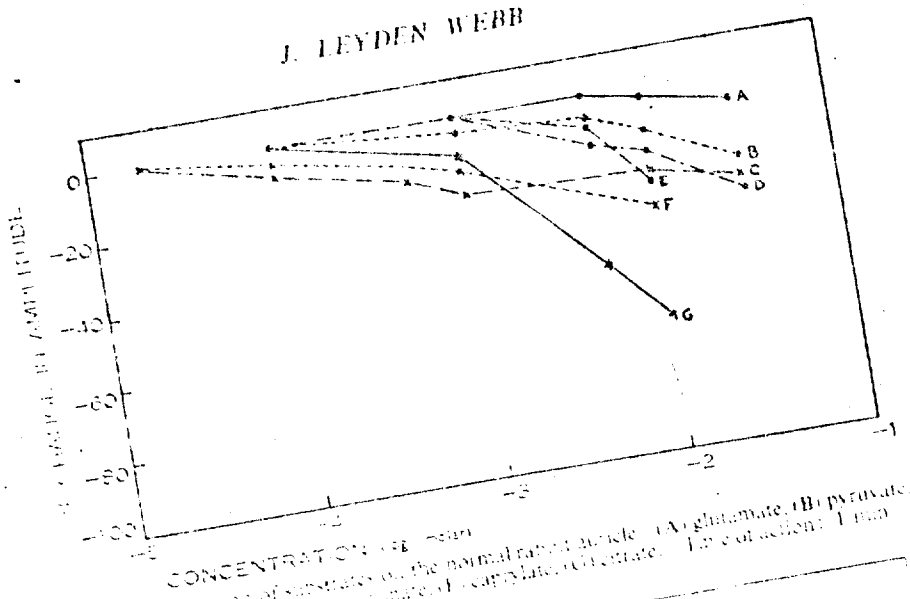

FIG. 1.—The action of substrates on the normal rabbit auricle. (A) glutamate, (B) pyruvate, (C) acetate, (D) malate, (E) succinate, (F) caprylate, (G) citrate. Time of action: 1 min.

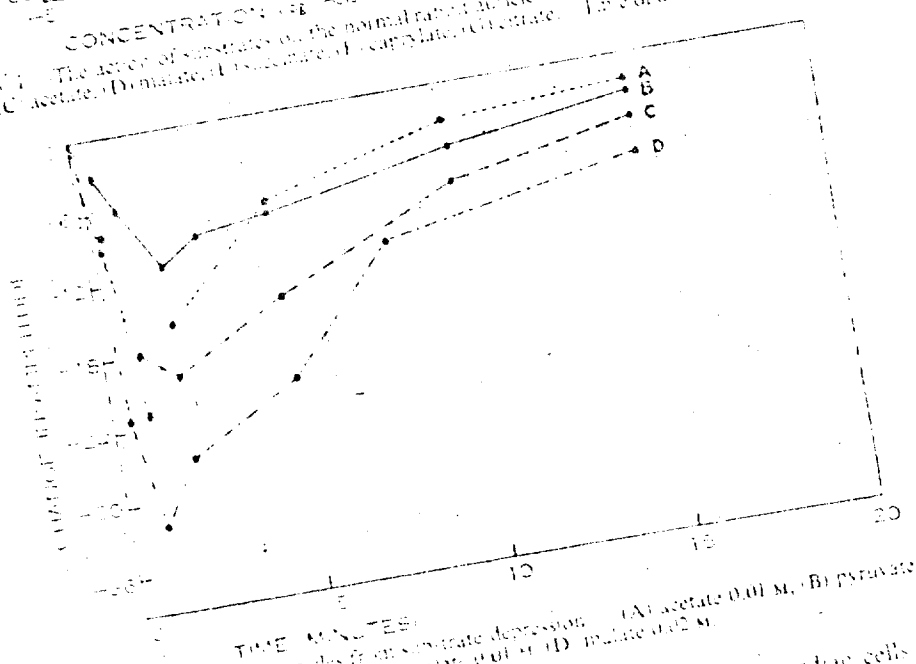

FIG. 2.—Recovery of auricles from substrate depression. (A) acetate 0.01 M, (B) pyruvate 0.005 M, (C) malate 0.01 M, (D) malate 0.02 M.

Consideration of the penetration of these substances into the cardiac cells is of the greatest importance, for, unless the heart cells, like some bacteria, possess enzymatic systems upon their surfaces, it is necessary for the substances to enter the cells in order to produce a direct effect upon the metabolism. Very little work has been done on the permeability of cell membranes to such substances. Further-

more, no analogies between the penetration of these substances and other weak acids can be drawn with confidence, inasmuch as cell membranes seem frequently to possess selective permeabilities for substances of functional importance. At the slightly alkaline pH of this medium, these substrates exist in solution preponderantly as anions; this may interfere with rapid penetration, especially when the substrates possess two or three such ionized groups. However, it must be borne in mind that the permeability to charged molecules may markedly increase in the heart cells during the passage of an impulse over the tissue. There is some evidence for the penetration of such substrates. It will be shown later that in substrate-depleted auricles there is an almost immediate effect of added pyruvate. Furthermore, the actions of pyruvate, lactate, β-hydroxybutyrate and other substrates are quite rapid on exhausted perfused hearts. It will also be shown that iodoacetate and fluoroacetate penetrate the auricular cells quite readily. With regard to dicarboxylic and tricarboxylic acids, I know of no evidence that is applicable to the present situation, although it seems certain that succinate and malonate are able to penetrate into heart slices. Fig. 1 shows no correlation between effect and the number of carboxyl groups. I believe that the evidence available points to a definite penetration by these substances. However, equilibrium may not be reached for one to three minutes, the times of maximum substrate action thus being expressive of the relatively slow rate of penetration compared to uncharged molecules.

It is possible that the effects observed with the substrates may have been partly or wholly non-metabolic effects. The relationship between cellular membrane potentials and the rate of discharge of the pacemaker cells or the degree of contraction is not understood, but it is conceivable that alterations brought about in the cell membrane by these substrates might influence the auricular functioning. Such organic acids are known to be capable of producing temporary electrical potential changes in nerve, muscle and plant cells. If such a mechanism of action is involved, it is likely that we must interpret the spontaneous recoveries from substrate depression as originating in the gradual restoration of the normal potential by the establishing of a concentration equilibrium between the medium and the interior of the cells. That these substrates depress the auricles by increasing in some manner the effective acetylcholine concentration in the tissue is unlikely, since it was clearly shown that atropine, at a concentration capable of blocking all acetylcholine action (1×10^{-6}), failed to alter the actions of pyruvate, citrate, acetate, malate, and fumarate. It is improbable that the depressant action originates in a simple increase in osmotic pressure of the medium, for glucose may be added up to a concentration of 0.03 M without alteration of the beat. Furthermore, if an osmotic effect were involved, we should expect the various substrates to produce similar degrees of depression at equivalent concentrations; this, however, is not the case. Since the concentration-action curves for the various substrates, as shown in Fig. 1, are not only at different positions along the concentration axis, but also possess markedly different curvatures, it would seem unlikely that the depressant effects were due to any general, non-specific type of action.

Some of the substrates, particularly acetate, malate, and propionate, showed a tendency to induce arrhythmias in the auricles. These arrhythmias were occasionally completely irregular, but usually they were of the "pulsus alternans" type, the amplitude of contraction regularly alternating each beat between two different

values. Sometimes such a rhythm was caused by the regular beating of one auricle of a pair while the other auricle beat only every other contraction, but more frequently this was not so. Rapid recoveries from such arrhythmias were observed when the substrate was removed from the bath.

The action of succinate was only depressant in these experiments. Depression of the amplitude was observed by Salant, Livingston, and Connet (1917) on the perfused frog heart (succinate 0.01–0.02 M) and by Forssmann and Lindstén (1946) on the isolated and perfused rabbit heart (succinate 0.01–0.04 M). They did not find in these preparations such marked depression of the rate as was here observed on the rabbit auricles. Forssmann and Lindstén (1946) found a mild stimulation (+15 per cent) of the amplitude by succinate in lower concentrations (0.0005–0.005 M). In the auricles no such stimulation was observed within a similar concentration range; this possibly indicates a difference in behaviour between the auricles and ventricles of the rabbit, inasmuch as they measured the contractions of the left ventricle. No stimulation was found in the isolated and perfused rat heart (Nakamura *et al.*, 1949). Despite the rapid oxidation of succinate by cardiac tissue, it would appear that the energy derived from this is, at least in the normal auricle, of no consequence in the functioning of the auricles. It will be shown later that this energy cannot be utilized to revive substrate-depleted auricles.

The relatively marked depression produced by malate on the auricles is in accord with the results of Salant, Livingston, and Connet (1917) on the frog heart; the frog heart would seem to be more sensitive to the action of this substrate than the rabbit auricle. A definite difference in the behaviour of the two tissues is seen, however, in the recovery of the rabbit auricles from the malate depression, whereas the frog heart was reported to remain in a constant state of depression as long as the malate was present (in one case for 62 minutes).

The action of citrate on the rabbit auricle is very interesting, there being a marked stimulation of the rate while the amplitude is simultaneously reduced. This phenomenon occurs only at higher concentrations of citrate (above 0.005 M); below this there is little effect on the rate, although the amplitude is depressed. The acceleration of the rate occurs quite suddenly. For example, in one experiment using 0.01 M-citrate, the rate was unchanged 30 seconds after the addition of the citrate, but it suddenly increased by 22 per cent at 40 seconds, and was up by 39 per cent at 60 seconds, after which it remained relatively constant. The depression of the amplitude observed here is very similar to that reported by Forssmann and Lindstén (1946) on the entire heart, but, unfortunately, they did not include their results on the rate changes. How much of the action of citrate was due to a decrease in the concentration of ionized calcium in solution is difficult to say. Added calcium will counteract the depression produced by citrate, but this does not prove that citrate acts by calcium depletion. When the concentration of citrate is 0.001 M or above, there must be a decrease in the ionized calcium, the original concentration of the calcium ion in the medium being 0.0018 M. If one considers the effect on the rabbit auricle of decreasing the calcium concentration (Webb, 1950), and compares this with the concentration-action curve for citrate depression of the amplitude, one must admit that the results coincide with what one might expect if citrate were acting by calcium depletion. On the other hand, the stimulation of the rate cannot be due to a decrease of ionized calcium in the medium, for low calcium concentra-

tions have very little, if any, effect on the rate of the rabbit auricle. There still remains the possibility that the citrate penetrated the pacemaker cells and within them produced a disturbance in the calcium equilibrium, thereby producing changes in discharge rate that would not have occurred as the result of a mere decrease in external calcium.

The effect of glucose deficiency on rabbit auricles

If rabbit auricles were left for 24–48 hours at 30° C. in a glucose-containing medium a marked reduction in the amplitude was usually observed, especially if the medium had been continually replaced, although the rate often remained the same as it was originally. Under such circumstances the auricles stop in the relaxed condition of diastole. These auricles could usually be restarted with acetylcholine or adrenaline (Vane, 1948; Burn and Vane, 1949) but not by any of the substrates that were tried (pyruvate, acetate, succinate, oxalacetate, and malate). This type of exhaustion occurs in the presence of glucose and is probably not related in any way to depletion of substrate. It is likely that some alterations of the cellular enzyme systems have taken place, such as the depression of the formation of acetylcholine (Bülbring and Burn, 1949) or a dissociation of the reactions of oxidation and phosphorylation.

If glucose was removed from the medium in which the auricles were beating, there was no immediate effect. It is to be expected that the auricles would have sufficient endogenous substrate—glycogen and fat—on which to operate for a period of time. The amount of work performed by beating auricles under these conditions was not great, for they were not working against the usual internal pressure of the blood. Seven experiments were performed in which the behaviour of the auricles in the absence of glucose was observed for five to seven hours. In three of these experiments there was no change of rate, and in four there was some decrease (–10 to –30 per cent). In three of these experiments there was no change in amplitude, and in four there was some decrease (–30 to –50 per cent). The auricles thus show variation in their behaviour in the absence of glucose, some apparently having a greater supply of endogenous substrate than others. Since there was essentially no change in rate or amplitude over a period of five to seven hours in the presence of glucose, these decreases in rate and amplitude, when they occurred, must be attributed to the lack of glucose. In longer experiments, when the auricles were left without glucose overnight, the exhaustion produced by substrate depletion is complicated by the simultaneous development of the type of exhaustion seen when glucose is present. Furthermore, it seems evident that lack of glucose in the medium produced some irreversible change or damage in the cardiac cells, for although stimulation was observed when glucose was added to such auricles, there was never anything like complete recovery. This type of damage may be related to that occurring when cardiac tissue is subjected to oxygen deficiency (Webb, Saunders, and Thienes, 1949a).

In order to produce rapid substrate depletion, with as little complication from other changes as possible, the auricles were driven with adrenaline (1×10^{-6}) in several experiments for varying periods of time in the absence of glucose. Such a procedure exhausted the auricles to a low level of activity in 15 to 60 minutes. If adrenaline were allowed to act for one to three hours, the auricles, after the

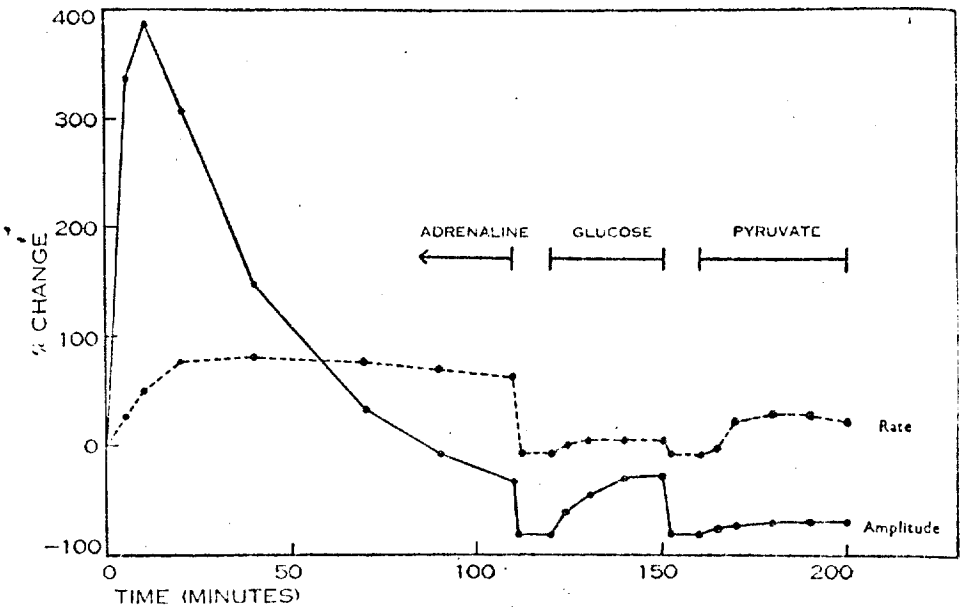

FIG. 3.—Substrate depletion of the auricles and the actions of glucose and pyruvate. Average of four experiments. Adrenaline, 10 °; glucose, 0.0055 M, pyruvate, 0.005 M.

adrenaline had been washed out, beat with a very small amplitude, although the rate was usually relatively unaffected. The averaged results of four experiments are shown in Fig. 3. The rate in the presence of adrenaline remained at a high level, while the amplitude rapidly decreased to below the normal level. After the adrenaline had been removed, the rate returned to nearly its original value and the amplitude was very depressed. Exhaustion with adrenaline in the absence of glucose seems to affect the amplitude much more than the rate, the latter being quite stable. In spontaneous exhaustion in the absence of glucose the amplitude was also more easily depressed. The question whether adrenaline produces cardiac failure by depletion of substrate under such circumstances has never been settled. Some investigators have found that adrenaline-driven hearts lose their glycogen and attribute the failure to this, while others have shown that the heart may fail when there is sufficient glycogen present (Chang, 1936; Fieschi, 1933; Bogue, Evans, and Gregory, 1937; and others). This problem might be solved by determining the extent to which the cardiac tissue will recover when glucose is given. If there is complete, or nearly complete, recovery the failure may be attributed to substrate depletion, while if no recovery is seen it is likely that damage and disorganization have occurred to the functional systems. Any intermediate situation would indicate the extent of substrate depletion or cardiac damage. In the present investigation we shall see that the situation is intermediate and we must conclude that changes have taken place in the auricular tissue in addition to substrate depletion. However, for convenience we shall speak of such auricles as depleted.

When the auricles were driven with adrenaline there was invariably a gradual development of contracture as the amplitude decreased. This may also be expressed as an increase in tone or a decreased ability of the auricular muscle to relax. Auricles brought to almost complete exhaustion with adrenaline in the absence of glucose usually beat at a normal rate with a greatly diminished amplitude and were in a state of contracture. This inability to relax was also seen in the longer experiments when glucose was absent and no adrenaline was used, but it was never observed in exhaustion occurring when glucose was present. The problem of relaxation will be discussed after the results obtained with various metabolic inhibitors have been presented.

Effects of metabolic substrates on substrate depleted auricles

Auricles that had been spontaneously depleted or driven with adrenaline in the absence of glucose were invariably stimulated by the addition of 0.0055 M glucose, the concentration normally present in Tyrode solution. Furthermore, the degree of contracture of the auricles was lessened; that is, the auricles showed an increased ability to relax. Sometimes the degree of relaxation could be alternately increased and decreased by additions and removals of glucose. Complete recovery to the initial amplitude was never seen, but the rate, if it was decreased, was frequently restored to its normal value. The rate, however, in the depleted auricles was never far below its original value, while the amplitude was markedly depressed to only 10-20 per cent of the initial height. Usually there was a fairly rapid rise in amplitude over the first two minutes after glucose addition, and this was followed by a more gradual increase to reach a maximum value after 10-40 minutes. In terms of percentage, glucose increased the amplitude by 100-300 per cent over the depressed value. This is illustrated in Fig. 3, where the action of glucose may be compared with that of pyruvate.

Pyruvate likewise stimulated the substrate depleted auricles but not so well as glucose. However, the effect upon the rate was more marked with pyruvate, the rate being occasionally increased over the normal original value, while a good effect was sometimes obtained on the rate with little or no stimulation of the amplitude. The stimulation of the amplitude by pyruvate was usually between 10-60 per cent, and when compared with glucose on the same auricles this amounted to about one-fifth of the glucose effect. Pyruvate also induced relaxation of the auricles but was not so effective as glucose in this respect. The optimal concentration of pyruvate for such stimulation was found to lie between 0.001-0.005 M.

Only acetate and β-hydroxybutyrate of all other substrates tested were capable of producing any recovery in the substrate depleted auricles, and these effects were small and variable. Neither of these substances was found to induce relaxation. With acetate, the concentration had to be low (0.001 M) for any stimulation to be observed, for if higher concentrations were used only further depression resulted. In six experiments with 0.001 M-acetate, the average increase in amplitude was 18 per cent whereas the rate was not stimulated. Substrates with no ability to revive depleted auricles were fumarate, succinate, citrate, malate, oxalacetate, propionate, butyrate, and glutamate.

It is interesting to note that glucose and pyruvate would occasionally facilitate relaxation of the auricular muscle although the auricles were not beating. Here we may confidently say that depletion of substrate increases the tone of the cardiac muscle and that the presence of glucose or pyruvate decreases the tone. When the auricles were beating, the amplitude of contraction usually decreased while the tone was increasing, and vice versa, but this was not always true, and we must conclude that there is no necessary relationship between the degree of contraction, and the ability of the muscle to relax. Failure of the muscle to relax sufficiently naturally inhibited the development of a full contraction, but it was observed occasionally, when glucose or pyruvate were added while the contraction was proceeding, that the amplitude may increase without simultaneous relaxation.

The results presented here on the rabbit auricle agree well with those obtained in the heart-lung preparation and in the isolated and perfused mammalian heart, not only with respect to the substrates that are utilizable by the cardiac tissue, but also with the comparative abilities of these substrates to revive the depleted heart muscle. However, on no other tissue are the differential actions of glucose and pyruvate on the rate and amplitude so easily observed. On may conclude that auricular and ventricular functional metabolism is qualitatively similar. It is likely, however, that somewhat different systems are involved in the discharge of impulses by the pacemaker cells and in the processes of contraction.

Effects of metabolic inhibitors on normal auricles

Curves showing the relationship between the concentration of inhibitor and the action produced are presented in Figs. 4 and 5. The time course of inhibitor

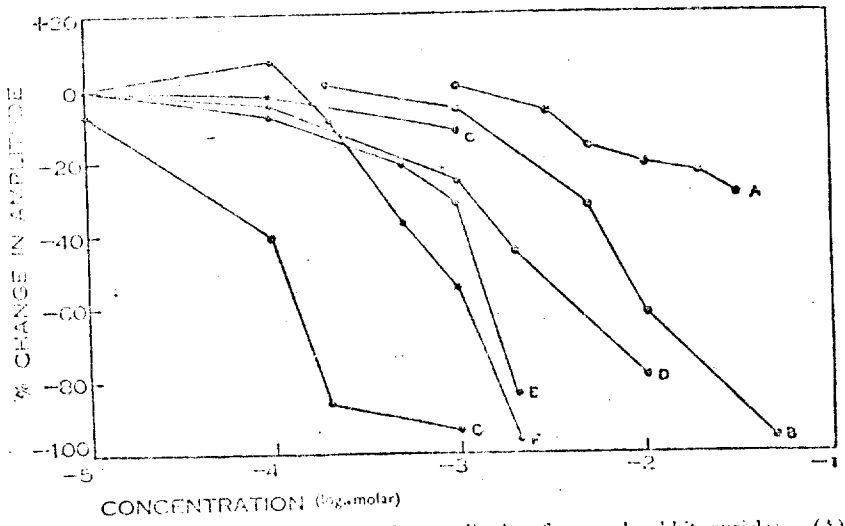

FIG. 4.—The action of inhibitors on the amplitude of normal rabbit auricles. (A) malonate—2 min., (B) fluoride—4 min., (C) phlorizin—5 min., (D) fluoroacetate—25 min., (E) azide—4 min., (F) cyanide—2 min., (G) iodoacetate—25 min. The times refer to the duration of inhibitor action.

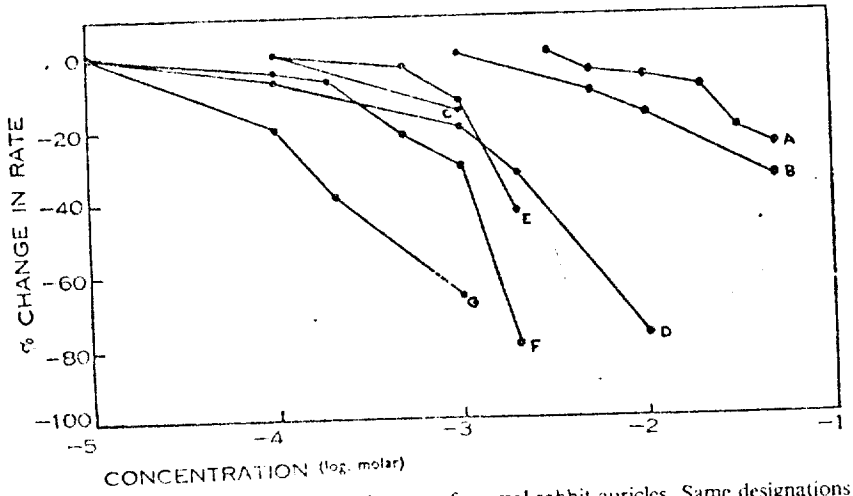

FIG. 5.—The action of inhibitors on the rate of normal rabbit auricles. Same designations as in Fig. 4.

action is illustrated in Figs. 6 to 12, where the actions upon rate and amplitude may be compared. These are all average curves obtained from three to six experiments.

Cyanide

Cyanide invariably produced a temporary stimulation before it began to depress the auricles, this stimulation being more marked in the amplitude than in the rate. The degree and duration of stimulation varied with the concentration of cyanide: at low concentrations (0.00001–0.0001 M) the stimulation was small but persisted, often not being followed by a depression, but as the concentration was increased the period of stimulation became shorter and shorter, so that at concentrations around 0.001 M the maximum stimulation was reached 20–40 seconds after addition and was followed immediately by a rapidly developing depression. Such a temporary stimulation was also seen when the auricles were made anoxic.

The rapid development of the depression would indicate that the available supply of high-energy phosphate bonds in the auricle, at least with respect to some process, is not great, unless cyanide, through some unrecognized action, prevented the direct utilization of this energy. A concentration of cyanide of 0.001 M produces a final depression of about 90 per cent in the amplitude and 50 per cent in the rate; this concentration was reported to inhibit the respiration of minced rabbit heart 45 per cent (Banga, Schneider, and Szent-Györgyi, 1931) and the respiration of rat heart slices 74 per cent (Webb, Saunders, and Thienes, 1949b). The rabbit auricle will usually stop in a concentration of 0.002 M-cyanide. It is probable that, when the auricles stop, there is still a definite fraction of the respiration proceeding; the level of activity to which the auricles are brought by any concentration of cyanide is not a quantitative measure of the cyanide-resistant respiration. Impulse discharge from the pacemaker cells was not inhibited so readily as the amplitude of contraction; this has been observed with other cardiac

preparations (Loewi, 1897; Beresin, 1913; and others). Most workers have reported that hearts poisoned by cyanide stop in diastole (Loewi, 1897; and others) but some have observed systolic standstill (Fujimaki, 1924). In the rabbit auricle both may be observed according to the conditions of the inhibition. When the auricles were stopped rapidly by higher concentrations of cyanide, they stopped in the relaxed condition of diastole; however, if the action of cyanide at concentrations just below those required to stop the auricles was prolonged, the auricles began to go into contracture and eventually stopped in systole. Occasionally both phenomena were observed in the same experiment. In one instance, the auricles were stopped in 80 seconds by 0.002 M-cyanide, but they slowly and partially recovered in the presence of cyanide, so that at 30 minutes the rate was depressed to only 40 per cent of its original value and the amplitude 92 per cent of its original value. At this stage the auricles were in a state of partial contracture, an inability to relax having been developed during this time. Eventually they stopped in systole. However, contracture is less commonly observed with cyanide than with inhibitors like iodoacetate, fluoroacetate, and fluoride.

Spontaneous recovery from depression in the presence of cyanide was very seldom observed, the example cited above being the only definite instance. Normally the auricles would reach a certain level of depression and remain there, or, if the depression were sufficient, they would slowly pass into a state of contracture. The auricular tissue seems to have very little ability to adjust itself to conditions in which the oxygen utilization is impaired; no ability to adjust to anoxic conditions was observed. During very slight cyanide depressions, there was occasionally some recovery, but this may be explained by loss of cyanide from the medium, some being carried off by the vigorous gassing. When the cyanide was removed from the medium, the auricles recovered completely, the rapidity of recovery depending on the concentration of cyanide and the duration of exposure. After marked depression following exposures of three to six minutes to 0.001 M-cyanide, the auricles would recover completely within ten minutes, but if the exposure time was only one minute the recovery took place within three to five minutes. No irreversible damage to the cells was observed at these concentrations, unless full contracture was allowed to occur. After the cyanide had been washed out a stimulation of the rate during the recovery phase was frequently observed, amounting to a 20–30 per cent increase over the normal rate before the cyanide had been added. Various arrhythmias commonly occurred during this recovery phase. It is possible that during the depression some energy-containing or energy-supplying substance had accumulated in the cells and that when oxygen was again available this resulted in a temporary stimulation of activity; this stimulation, however, was observed only with regard to the rate.

When excess calcium was added to the medium there was a marked stimulation of the amplitude, the rate remaining unchanged (Webb, 1950). Cyanide (0.001 M) antagonized this action, so that lower concentrations of calcium (0.0001–0.0005 M) were almost without effect, while the action of higher concentrations was markedly diminished. Cyanide also diminished the stimulating action of adrenaline on the auricles. In one experiment, adrenaline (10^{-6}) normally increased the rate by 69 per cent and the amplitude by 390 per cent, but in the presence of cyanide (0.001 M) the observed effects of adrenaline were zero and 105 per cent respectively.

Azide

It is interesting to compare the actions of cyanide and azide because of the usually accepted similarity of their enzymatic inhibitions. Azide was not as potent an inhibitor of the auricles as cyanide. In equal concentrations, azide acted more slowly than cyanide and at any particular time produced only 1/2 to 1/3 the depression that would have been produced by cyanide. In Figs. 6 and 7 comparison may be made of their actions at 0.0005 M. Azide did not produce the temporary stimulation observed with cyanide, nor did low concentrations ever stimulate. As with cyanide, azide depressed the amplitude more than the rate. This was also observed on the isolated hearts of cats and rabbits by Graham (1949).

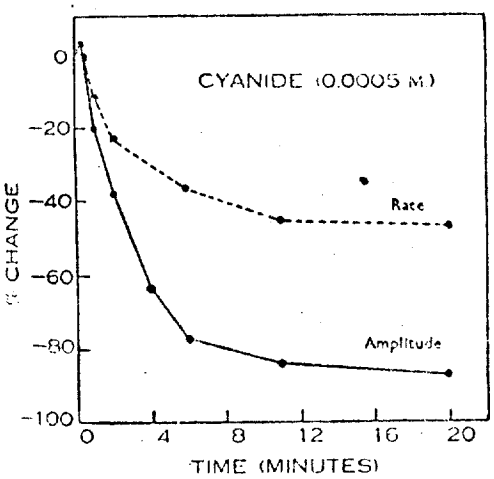

FIG. 6.—The action of cyanide on the rabbit auricle.

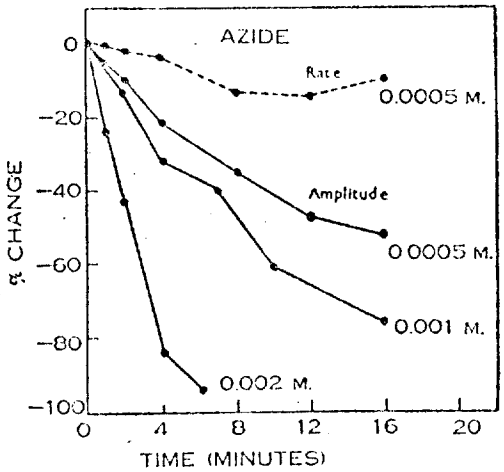

FIG. 7.—The action of azide on the rabbit auricle.

Recovery of the auricles after the azide had been removed was slower than with cyanide; 10 minutes were required after an exposure of 12 minutes to a concentration of 0.0005 M, and 20 minutes after a four-minute exposure to a concentration of 0.002 M, indicating that the azide is more tightly bound than cyanide.

Armstrong and Fisher (1940) concluded that cyanide and azide acted differently on fish hearts because the cyanide-stable respiration was different from the azide-stable respiration. However, this may be merely an expression of the wider range of action of cyanide at higher concentrations, for it is known that cyanide above a concentration of 0.001 M begins to inhibit dehydrogenases in addition to its action on metal-containing enzymes. However, it is clear that even in lower concentrations there was some difference in behaviour between the two inhibitors in their actions on the rabbit auricle.

Fluoride

Fluoride at concentrations between 0.005–0.01 M first depressed both the rate and amplitude of the auricles, but after approximately five minutes the amplitude began to increase and eventually reached values above the normal original level

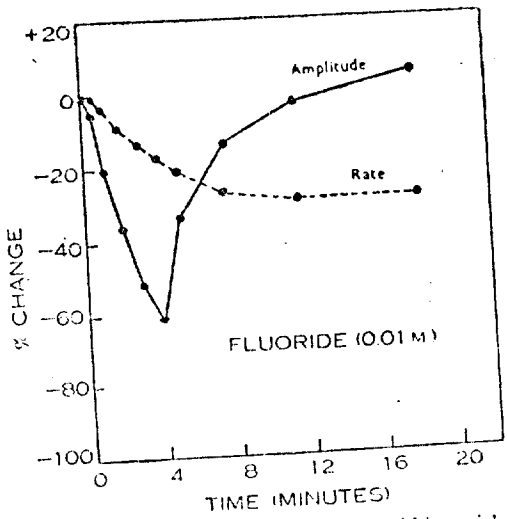

FIG. 8.—The action of fluoride on the rabbit auricle.

(see Fig. 8). Meanwhile, the rate remained at its depressed level or continued to decrease slightly. After 20–40 minutes the auricles were beating slowly but with a large amplitude. However, occasionally contracture began and the amplitude again decreased owing to the increasing inability of the auricles to relax. Previous reports on the action of fluoride on cardiac tissue have been equivocal. Several investigators have found only depression: Vernon (1910a, b) with 0.24–1.2 M-fluoride on the perfused tortoise heart, De Nitto (1928) with 0.00012–0.0012 M-fluoride on isolated mammalian hearts, and Chang (1938) with 0.0078 M-fluoride on rabbit auricles. Gottdenker and Rothberger (1935a, b) found depression of the perfused frog heart and the dog heart-lung by fluoride, but on guinea-pig and rabbit auricular strips they usually observed an increase in the amplitude when the fluoride concentration was 0.003 M. Ferrannini (1936) also found fluoride (0.0024–0.0072 M) to increase the force of contraction of rabbit auricular strips. In most work it has been observed that cardiac tissue is able to function surprisingly well in the presence of fluoride at concentrations which should inhibit glycolysis completely. Chang (1938) stated that fluoride, except for a slight depression, had little effect on the mechanic ability of rabbit auricles over a period of two to three hours as long as oxygen was present. Furthermore, he apparently observed no increases in amplitude. In the present work with the same tissue such a concentration of fluoride produced a marked effect. No explanation can be offered for these discrepancies.

With regard to the mechanism of fluoride action, most workers have concluded that depletion of calcium from the medium is not important. However, at least in the present experiments, it is difficult to see how it could fail to be of some importance. The calcium concentration in Tyrode solution is 0.0018 M; if sodium fluoride is added to the medium in a concentration of 0.005 M, there are approximately equivalent amounts of calcium and fluoride to form CaF_2. Since CaF_2 is soluble to the extent of 0.00022 M and since it is very little ionized (solubility product is 3.4×10^{-11}), it would seem likely that a definite fraction of the calcium would be withdrawn from the medium. When more fluoride is added the calcium should be reduced even more. No visible precipitation of CaF_2 was observed, but the walls of the tissue bath slowly became covered with a white layer. Reduction of calcium in the medium leads to a marked depression of the auricles with respect to amplitude, but has no effect on the rate (Webb, 1950). Thus it would seem that the observed depression of rate and the subsequent increase in

amplitude, in the presence of fluoride, must have been due to an action of fluoride other than that of altering the calcium concentration in the medium. As to how much the initial depression of amplitude is due to decreased calcium it is impossible to say.

Since fluoride inhibits cholinesterase, the possibility exists that at least part of the depressant action was due to prevention of destruction of endogenous acetylcholine. However, fluoride was found to give a typical depression of the auricles in the presence of physostigmine (10^{-7}), when the cholinesterase was already inhibited, and also in the presence of atropine (10^{-5}), when the action of acetylcholine was completely prevented. It is thus unlikely that the action of fluoride is related to the acetylcholine equilibrium in the auricles.

Such concentrations of fluoride as were found to have definite actions on the auricles (0.005–0.05 M) should inhibit glycolysis completely. Muscle enolase is usually inhibited 50 per cent by 0.001 M-fluoride and completely by 0.005 M-fluoride. A certain decrease in fluoride concentration would result from combination with calcium, but in the higher concentrations this could not have been very great. We must, therefore, conclude that the auricles can function quite well in the absence of the formation of phosphopyruvate from 2-phosphoglycerate. One possibility is that the auricles used fat for the necessary energy. Another possibility is that the 2-phosphoglycerate was utilized through some other system without the formation of pyruvate. It is interesting to speculate that the secondary increase in amplitude is due to this new source of energy, brought into play because of the enolase block. In rat heart slices the respiration was never depressed more than 42 per cent by fluoride at concentrations as high as 0.1 M (Webb, Saunders, and Thienes, 1949b), so it is evident that in cardiac tissue there is an appreciable fraction of the metabolism insensitive to fluoride.

Fluoride inhibition is reversible, and after the fluoride had been washed out recovery was usually complete within 5–10 minutes. After long exposures there was occasionally some stimulation of the rate above the normal figure during the post-wash recovery stage. It was also noticed that the auricles during this period frequently had difficulty in relaxing and the tone varied correspondingly. In passing, it may be remarked that fluoride (0.03 M) quite effectively diminished the action of adrenaline (10^{-7}–10^{-6}), the stimulation of amplitude being almost abolished while the rate effect remained but was made weaker.

Iodoacetate

Iodoacetate in concentrations between 0.0001–0.001 M is a slowly acting depressant of the rabbit auricle, requiring from three to five minutes for appreciable reduction of the amplitude and from 10 to 20 minutes for a slowing of the rate. At certain stages of the depression, the amplitude was markedly reduced while the rate remained unaffected, but eventually both were depressed almost equally before the auricles stopped. During the later stages of this depression the auricles began to go into contracture and they stopped in systole, a phenomenon that has been invariably observed in work with iodoacetate (Weicker, 1934; Chang, 1938; and others). Although slight depressions produced by low concentrations of iodoacetate acting for short periods of time could be partially reversed by removing the iodoacetate, the usual result was that after the iodoacetate had been washed out the inhibition

proceeded, exactly as if the inhibitor were still present in the medium. This is, of course, what one would expect from considerations of the irreversible cellular reactions of iodoacetate, and it confirms previous results on other tissues and preparations.

Iodoacetate is not a specific inhibitor of glycolysis in mammalian cardiac tissue (Webb, Saunders, and Thienes, 1949b), and even in low concentrations it is likely that other enzyme systems are involved in its action. Not only is the synthesis of ATP diminished, by inhibition of the utilization of pyruvate and fatty acid breakdown products as well as by prevention of glycolysis, but it is likely that iodoacetate interferes with the utilization of ATP, for Buchtal, Deutsch, and Knappeis (1943) have reported that iodoacetate blocked the stimulating effect of ATP on skeletal muscle. It would not be surprising if iodoacetate reacted directly with myosin, since this protein complex possesses a number of sulphhydryl groups important to its functioning. This may explain the observation (Chang, 1938) that there is some ATP present in rabbit hearts that have been stopped by iodoacetate.

Adrenaline produced very little stimulation of the iodoacetate depressed auricle, and occasionally a reversal of the rate effect was observed when the depression was well advanced. In one typical experiment the concentration of iodoacetate was 0.001 M, the exposure time was 35 minutes, and the auricles were markedly depressed (rate −42 per cent, amplitude −96 per cent); adrenaline (10^{-6}) stimulated the amplitude slightly (28 per cent), but the rate was decreased to −75 per cent of the original value. It is not surprising that such auricles would fail to be stimulated by adrenaline, but the reversal of the rate effect was not expected. It is possible that iodo-acetate blocks some step in the series of reactions through which adrenaline normally produces a stimulation of the rate, leaving the systems by which it may depress the rate at higher concentrations relatively unaffected.

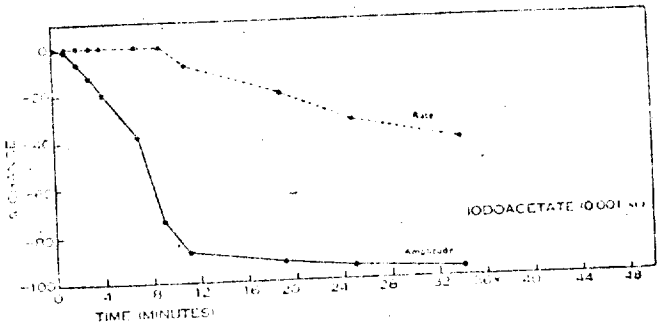

FIG. 9.—The action of iodoacetate on the rabbit auricle.

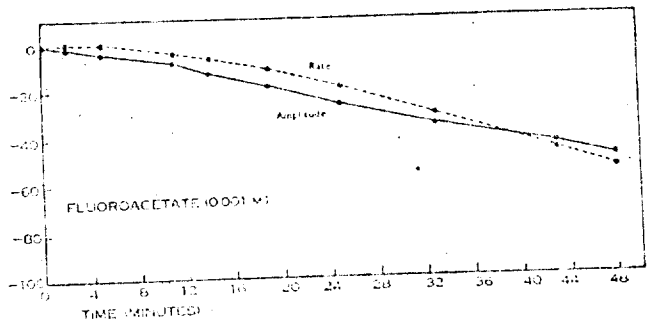

FIG. 10.—The action of fluoroacetate on the rabbit auricle.

Fluoroacetate

Fluoroacetate is the most slowly acting inhibitor of those investigated, depressing the auricles gradually and steadily for a period of two to three hours at a concentration of 0.001 M. It characteristically depressed both rate and amplitude equally, in this respect differing from any other inhibitor used in this study. The metabolic systems involved in impulse discharge and contraction may at some point possess a common path which is attacked by fluoroacetate. At the present time the exact mechanism of action of fluoroacetate is unknown, but it is likely that it inhibits some reaction, or reactions, in the tricarboxylic acid cycle. Accumulation of citrate and acetate during fluoroacetate action has been reported, but the depression of the auricles was not due to these intermediates, as may be seen by comparing the action of fluoroacetate with the actions of these substances. Furthermore, if such were the explanation, we should expect some degree of recovery when fluoroacetate was removed from the bath, but this did not occur. The action of fluoroacetate was quite irreversible. Occasionally the inhibition could be arrested by washing out the inhibitor, but the auricles never showed any tendency to return to their normal rate and amplitude.

Phlorizin

A nearly saturated solution of phlorizin in the medium (0.001M) produced a slight depression of the auricles, the effect on the rate being somewhat greater than on the amplitude, the latter showing some ability to recover spontaneously in the presence of the inhibitor. The mechanism of action of phlorizin at these higher concentrations is not well understood, and therefore it is difficult at the present time to interpret these results or correlate the actions with any particular inhibition of enzymatic systems.

Pyrophosphate

Pyrophosphate at concentrations between 0.002–0.005 M produced a marked depression in the amplitude of the auricles but had little effect on the rate, slight

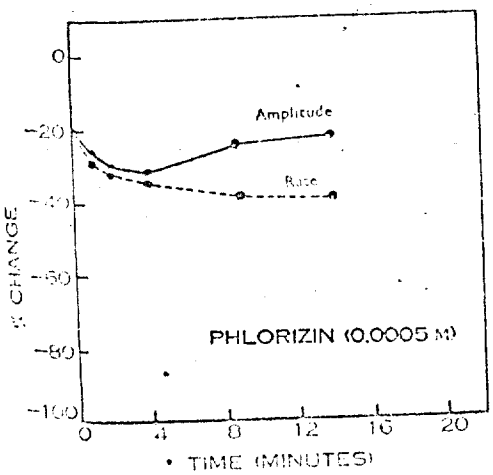

FIG. 11.—The action of phlorizin on the rabbit auricle.

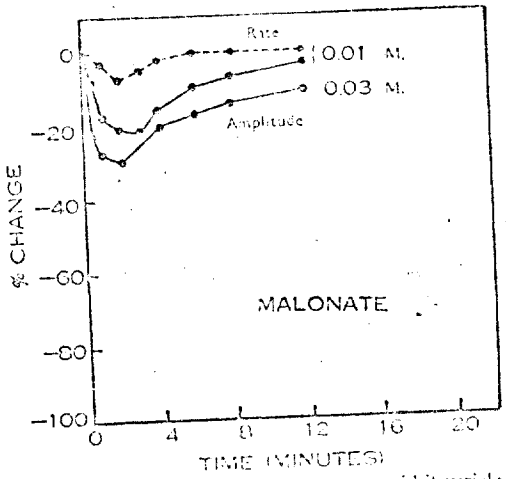

FIG. 12.—The action of malonate on the rabbit auricle.

stimulation occasionally being the only effect observed. Since pyrophosphate combines with calcium and the medium became slightly turbid after its addition, the possibility was considered that the action upon the auricle was due to a decrease of ionized calcium in the medium. If calcium was added to the medium during pyrophosphate depression the amplitude returned to normal, but the increased rate was unaffected. Lack of calcium in the medium leads to depression of the amplitude, but the rate is never stimulated and is occasionally slightly reduced. Recovery of the auricles after low calcium was more rapid than after pyrophosphate depression. We may conclude, therefore, that the effects produced by pyrophosphate were partially due to a lack of ionized calcium, but that the pyrophosphate also had a definite action of its own. Since these actions could not be separated conveniently, the action of pyrophosphate was not studied farther and the curves of its action are not included in the Figures.

Malonate

The rabbit auricle was not strongly depressed by malonate; at high concentrations (0.02–0.03 M) a maximum inhibition of only 30 per cent in rate and amplitude was reached. Furthermore, the auricles spontaneously recovered in the presence of malonate at these high concentrations and returned to normal, or nearly normal, rates and amplitudes after 8–10 minutes. When the malonate was washed from the bath during the period of greatest depression, the recovery was very rapid, requiring only two to three minutes. Forssmann and Lindsten (1946) found that malonate in concentrations between 0.01–0.04 M depressed the ventricular contractions of the perfused rabbit heart but had little effect on the rate. It is remarkable that malonate at these concentrations should have so little depressant action on the auricles, for it is generally believed that malonate at a concentration of 0.005 M completely inhibits succinic dehydrogenase and therefore blocks the tricarboxylic acid cycle, a system upon which the cardiac muscle is thought to depend. Recently it has been reported that somewhat higher malonate concentrations, such as those used here, will prevent the oxidation of oxaloacetate in homogenates, this action producing an additional derangement of the tricarboxylic acid cycle (Pardee and Potter, 1949). It seems unlikely that failure to penetrate the muscle cells could have been responsible for this lack of action, inasmuch as exposures of 20–30 minutes to malonate did not lead to any marked alteration of auricular function. The similar behaviour of cardiac cells under the influence of malonate was noted previously on rat heart slices (Webb, Saunders, and Thienes, 1949a). In this preparation malonate produced a minimal depression of the respiration, but this was followed by a recovery to values near the normal rates. Furthermore, in the rat heart slices it was known that the succinic dehydrogenase was inhibited, for no rapid increase in oxygen uptake was observed when succinate was added as invariably happened when malonate was absent. Rat heart slices can respire adequately in the presence of high concentrations of malonate, and auricles can function quite normally under these conditions. We must conclude that cardiac tissue can respire and function when this particular step in the tricarboxylic acid cycle is blocked. It may well be that when this block is imposed in an organized cellular system, the cycle can be short-circuited; perhaps under such conditions oxaloacetate may be formed directly from α-ketoglutarate, or pyruvate or its activated fragment be taken into the

cycle by combination with something other than oxaloacetate. It will be shown later that the small amount of depression observed with malonate is probably not due to the inhibition of succinic dehydrogenase.

Interactions of substrates and inhibitors on the auricles

Experiments were performed to investigate the abilities of various substrates to counteract or antagonize the depressions produced by metabolic inhibitors. Theoretically, if an inhibitor acts specifically on one enzyme in a series of enzymic reactions, the addition of the intermediate substrate that is normally formed by the action of this enzyme should allow at least partial recovery of the functional activity of the tissue. If the inhibitor acts not only on this one enzyme, but upon others as well, the amount of recovery produced by the added intermediate will be roughly proportional to the specificity of the inhibitor effect. The possibility must always be considered that an added substrate, in the presence of an inhibitor, may initiate energy-forming reactions that are not normally functional; the stimulation of the auricles by a substrate during an inhibitor depression does not necessarily mean that there is a direct relationship between the inhibitor and the substrate in the cellular metabolism.

The relatively weak effect of malonate on the auricles was puzzling, and attempts were therefore made to determine whether the depression that was produced was related to the commonly accepted action of malonate on succinic dehydrogenase. Malonate prevents the formation of fumarate from succinate, and therefore from malonate depression should, therefore, be observed after the addition of fumarate. If malonate acted simultaneously elsewhere in the tricarboxylic acid cycle this would not necessarily be true. Three types of experiment were performed.

(1) In two experiments the effects of malonate (0.01–0.03 M) were determined, and these were compared with the effects of adding malonate and fumarate (0.001–0.01 M) together. No modification of the action by fumarate was observed. In a typical experiment the average maximum depression of amplitude produced by malonate alone was 25.7 per cent; when both malonate and fumarate were added together the depression was 27.7 per cent. The changes in rate were likewise comparable.

(2) In the second type of experiment fumarate (0.001–0.01 M) was added to auricles already depressed by malonate (0.02 M). No stimulating effect was observed; in fact, an additional depression was usually seen.

(3) In the third type of experiment the auricles were allowed to remain in contact with fumarate (0.005 M) for five minutes before the addition of the malonate (0.01 M) in order to ensure penetration of the fumarate. Again no change in the response to malonate was observed.

It therefore seems likely that the malonate depression is not due solely to inhibition of succinic dehydrogenase. It is more reasonable, I believe, to assume that malonate at such concentrations exerts a toxic action on the cell surface, a depression such as is seen with any of such substances under similar conditions. Acetate (0.001 M) and pyruvate (0.005 M) were also unable to counteract the depression produced by malonate (0.02 M), only additional depression being observed.

The depression produced by fluoride (0.005–0.02 M) was not counteracted by succinate (0.001 M), acetate (0.001 M), or citrate (0.001 M). However, pyruvate (0.005 M) showed some ability to stimulate the rate of the auricle in the presence of fluoride, but the amplitude was usually unaffected. This is in harmony with most of the results obtained with pyruvate, namely that it acts more particularly upon the mechanisms involved in rate than upon those involved in the amplitude of contraction. Complete antagonism of fluoride with pyruvate was never observed.

Iodoacetate, like fluoride, is generally supposed to act predominantly on the glycolytic stages of metabolism, inhibiting the formation of pyruvate from glycogen or glucose. However, there are reasons for supposing that in cardiac tissue the action is not specific. The heart muscle presumably obtains energy from both glycolytic and post-glycolytic phases of metabolism, although a relatively small [illegible] approximately 15 [illegible] per cent of the total). If iodoacetate [illegible] and restore the activity of the [illegible] value, only the energy from the [illegible] to the tissues. Three experiments were performed to test this.

(1) In the first experiment the concentration of iodoacetate was quite high (0.[illegible] M), the [illegible] been in contact with the inhibitor for 15 minutes, and the [illegible] per cent, amplitude [illegible] 95 per cent). [illegible] the rate and amplitude continu- [illegible] to develop. After the auricles were completely stopped at 30 minutes the addition of pyruvate was without effect.

(2) In [illegible] the concentration of iodoacetate was lower (0.[illegible] M) [illegible] 25 minutes and the auricles [illegible] per cent, [illegible] 87 [illegible] cent). The addition [illegible] had no effect on the amplitude. [illegible] the iodoacetate had reduced [illegible] minutes and to 24 in four minutes. [illegible]

[illegible] (0.0002 M) was allowed to act for 15 [illegible] trate [illegible] 19 per cent, amplitude [illegible] slightly [illegible] both rate and amplitude, [illegible] per cent in two minutes and the amplitude increasing by [illegible] seconds; these were the maximum effects and were only [illegible] of the auricle [illegible] the pyruvate had not been [illegible] depression pyruvate was [illegible] at both 0.001 M and 0.01 M [illegible]

[illegible] pyruvate is only partially able to counteract the depres- [illegible] the concentration of iodoacetate [illegible] observed. These experiments [illegible] a specific poison of the glycolytic [illegible] cardiac [illegible] Acetate (0.001–0.01 M) showed [illegible] to counteract the iodoacetate depression.

Depression of the [illegible] by fluoroacetate was [illegible] recognized by pyruvate. This [illegible]

(1) In the first [illegible] the time of exposure was [illegible] per cent, amplitude [illegible] per cent and the amplitude [illegible] its normal level and the amplitude to only [illegible] The addition of pyruvate at the same concentration [illegible] 3. [illegible] even greater stimulation, the rate increasing by 2[illegible] per cent and [illegible] 125 per cent in two minutes.

(2) In the second [illegible] for only 25 minutes and [illegible] per cent, amplitude [illegible] ing the rate [illegible] After [illegible] per cent [illegible] more [illegible] per cent. The effect on the amplitude was quite [illegible] minutes the auricle [illegible] in being progressively inhibited [illegible] the rate effect remained for a much longer time.

[illegible]

Each metabolic inhibitor [illegible] exert a unique and [illegible] one another with [illegible] of action, [illegible] relaxation, [illegible] presence of the inhibitor, and [illegible] removed. Each inhibitor can be distinguished [illegible] the rabbit auricle. The problem, however, [illegible] information on the mechanism of [illegible] data in conjunction with the [illegible]

The auricles may [illegible] (state of contraction). [illegible] them in a state of relaxation. [illegible] stop in systole, requires time for [illegible] in the action of any substance. There are substances which will rapidly arrest the auricles in diastole and, with continuing action, will induce contracture, the end result being systolic arrest. In the present experiments with rabbit auricles, the following have been found to lead to contracture: iodoacetate, fluoroacetate, fluoride, cyanide, anoxia, and substrate depletion. All of these would be expected to produce a state of reduced metabolic activity with a decrease in the amount of energy available to the muscle. This inhibition of the flow of energy would result in a gradual loss of ATP (and other substances containing high-energy phosphate bonds), such having been observed in the past under similar conditions. Simultaneously with the depletion of ATP the cardiac muscle becomes less and less able to relax, and when the ATP level becomes [illegible] and

will beat no longer. The ATP concentration need not drop to zero for this to occur. This conception is in harmony with the views of Szent-Györgyi (1947) on skeletal muscle, ATP being required for the relaxation of the muscle fibrils as well as for their contractile activity. The auricular muscle is in a low-energy state when it is contracted and in a high-energy state when it is relaxed. Adding glucose or pyruvate to a muscle in contracture would provide some ATP and the muscle would be able to relax and beat again.

Let us consider auricles that have been stopped in the relaxed state of diastole by some metabolic inhibitor. Why does the muscle not contract? There is [illegible] concentration of ATP. There is nothing fundamentally [illegible] for the muscle can be made to contract [illegible] such contractions for a period [illegible] depressed. The [illegible] mechanisms of contraction and [illegible] nature and serve to stimulate [illegible] induced by a wave of depolarization [illegible] which sweeps over the muscle [illegible] transformed into its low-energy [illegible] phases: the [illegible] of this impulse. [illegible] is [illegible] by the impulse. [illegible] reactions for their energy. Main- [illegible] require energy, and [illegible] of substances [illegible] excitatory mechanisms are [illegible] Occasionally a substance [illegible] the pacemaker [illegible] of conduction or [illegible] stimulated by [illegible] In many [illegible] occurred; [illegible] conservation of [illegible] dis- [illegible] to [illegible] of a certain [illegible] being [illegible] would lead [illegible] impulse, [illegible] amplitude of [illegible] than [illegible] being found [illegible] some fraction [illegible] taken place), [illegible] fraction of

all-or-none units contracting, or each unit in the muscle is contracting proportionately less. The solution to this problem is fundamental to cardiac physiology and pharmacology. Does cyanide, for example, when it inhibits the auricular amplitude by 20 per cent, prevent completely the contractions of 20 per cent of the contractile units, the remaining units contracting normally, or does it reduce the contraction of each unit an average of 20 per cent? This is not merely the old question of whether the concentration-action curve is an expression of the action of a substance on cellular processes or whether it is derived from the distribution of susceptibilities of the individual cells. A depressant may inhibit the excitatory reaction either by raising the threshold of response of the contractile units or by reducing the excitatory state that is initiated by the passage of the impulse. In the former case we should be measuring a distribution of susceptibilities, but in the latter case it would be a matter of a true action on a cellular process. There is no *a priori* reason why both actions may not be of importance, depending on the substance that is producing the depression. It should also be pointed out that, although the contractile units may normally behave in an all-or-none manner, it is entirely possible that various conditions may modify the degree of contraction.

How must we interpret the changes in amplitude brought about by the action of these metabolically important substances on the rabbit auricle? The results of the present investigation can be best explained by assuming that these substances modify the excitatory reaction and not the contractile mechanisms directly. Only when the cardiac muscle begins to go into contracture is the contractile mechanism itself altered. If this is the correct interpretation, it would seem that our conceptions of the actions of substances on cardiac muscle must be modified. Such an interpretation would also apply to the actions of drugs on the heart.

Throughout this investigation it was found that the changes in rate and amplitude of the auricles were relatively independent of each other. An increase or decrease in the amplitude had no particular effect on the rate, nor did changes in the rate, within limits, affect the amplitude. Quite frequently one would remain constant during a marked change in the other, and occasionally they would change in the opposite directions. The fundamental energy-forming enzymatic reactions may be quite similar in the processes that control rate and amplitude, but the paths of energy flow and utilization are different. It is true that most substances change the rate and amplitude in the same direction. This is perhaps more readily understandable when we consider changes in amplitude as arising, not from alterations in the contractile mechanisms, but from modifications of some cellular surface phenomenon that is involved in the excitatory reaction, such a surface state being related to the cellular membrane changes occurring in the pacemaker cells. In whatever way we consider the cyclical discharge of impulses, we must assume that there is the development of a depolarized state and that this is propagated over the cardiac muscle, this being responsible, directly or indirectly, for the excitatory reaction and the contraction of the myofibrils that lie in the region over which the impulse is passing. We can thus see a possible connexion between rate and amplitude, a relationship that implies no necessary simultaneous behaviour, but which, in the majority of cases, will lead to parallel

behaviour with respect to direction of change. However, the magnitudes of the changes in rate and amplitude need bear no relationship to one another.

Development of contracture was always a late process, following earlier alterations in the auricular rate and amplitude. We have supposed that this was due to a greater reserve of ATP or other substances containing high-energy bonds. It is indeed possible that the membrane phenomena of impulse discharge, conduction, and the excitatory reaction do not utilize ATP or similar substances, but that the cyclical processes involved are more directly dependent on the oxidative sequence of enzymatic reactions. Most of the metabolic inhibitors investigated, as well as substrate depletion and anoxia, depressed the amplitude more than the rate. This might be explained by assuming that the rate processes require less energy than those involved in the excitatory reaction, but this can hardly be the entire answer since there are instances in which the rate is depressed as much as the amplitude. The metabolic systems involved in the rate and amplitude may also be assumed to be somewhat different, and this is quite likely from a consideration of the characteristic actions of the various inhibitors, but it would explain only the differences in behaviour and not the basic fact that the rate is more resistant to change than the amplitude. Another explanation might be that the rate and amplitude mechanisms alter their functional level to different degrees in response to an equivalent change in some common factor. For example, if we assume that both the rate of discharge and the effectiveness of the impulse on the contractile units depend on changes in the degree of electrical polarization of the cell membrane, it is conceivable that a depression of the resting potential or a depression of the rate of repolarization would inhibit both processes. However, owing to the particular circumstances, the number of units reacting would be depressed more readily than the discharge rate. The excitatory reaction is probably quite delicately adjusted to the changes occurring at or near the cellular surface. This delicate balance assures a ready adaptability of the cardiac muscle.

We might expect, for the same reasons, that glucose and pyruvate would stimulate the amplitude more than the rate in substrate depleted auricles. This was found to be true for glucose, but pyruvate acted more particularly on the rate. Quantitative comparisons are complicated by the third action of these substrates, the stimulation of relaxation, an action somewhat more readily produced by glucose than by pyruvate. However, it was clear that pyruvate, both in substrate depleted auricles and in those poisoned by inhibitors, stimulated the rate more effectively than the amplitude and more effectively than it produced relaxation of the muscle. From this it might be concluded that the oxidations in the tricarboxylic acid cycle are the main source of energy for the rate processes. Supplying glucose does not lead to a sufficiently rapid formation of pyruvate, and therefore giving pyruvate directly has a greater stimulating effect. Why, however, is pyruvate less effective than glucose in stimulating the amplitude? The energy derived from the breakdown of glucose to pyruvate must be of greater importance to the processes involved in the contraction and relaxation of the muscle than it is to the rate processes. It is doubtful whether we are measuring the effect of either substrate on the excitatory reaction under such circumstances, the stimulation of ATP formation being the primary action in increasing activity of contraction. According to Szent-Györgyi (1947) the actomyosin requires so much ATP to

be active at all, aside from the ATP that is utilized for relaxation. Since ATP can be formed from energy derived from the breakdown of glucose to pyruvate it is not so surprising that glucose is somewhat more effective than pyruvate in this respect. However, in the cellular membrane processes of impulse discharge and the excitatory reaction the conditions are different; as we have seen, it is possible that these mechanisms do not require ATP and may operate directly from the oxidative systems, especially the tricarboxylic acid cycle. Since the tricarboxylic acid cycle can continue to operate in the metabolism of fat, the observation that the rate is more steady during inhibition of glycolysis (iodoacetate) than it is during inhibition of the tricarboxylic acid cycle (fluoroacetate) is explained. There remain, however, a number of problems- for example, the anomalous behaviours of malonate and fluoride- and these ideas are presented only as a working hypothesis.

The metabolic activities of contracted and relaxed cardiac muscle may be different. The metabolism may occur in cycles, in phase with contraction and relaxation, so that the conception of the metabolism as a steady state would be erroneous. Deductions based on steady state conditions would not necessarily be applicable, nor would arguments based on limiting reactions always be valid. This must be kept in mind in considerations of the action of metabolic substrates and inhibitors on the heart. Determinations of the ability of heart slices to oxidize various substrates may give information on only one phase of the cycle; it is likely that such slices are in a state of contracture. The same reasoning would be applicable to the actions of inhibitors. Substrates that are well oxidized by the contracted muscle may produce no stimulation of the normally beating auricle because their oxidation occurs at phases of the cycle where their energy cannot be utilized; only when the cardiac muscle has become abnormal, when these cyclical processes have become distorted, or when the muscle has gone into contracture will these substrates be oxidized with the production of utilizable energy. Similar metabolic cycles of activity probably occur in the pacemaker cells during rhythmic discharge of impulses and near the surfaces of the muscle cells as the propagated impulses pass.

Furthermore, one is not justified in assuming that there is a general synthesis and supply of ATP upon which all the processes in the cardiac cell must draw. ATP is probably used in only certain functional mechanisms and is synthesized in these particular regions. The concentration of ATP as determined by analysis of the entire muscle can seldom be correlated with the functional activity of the tissue for this reason. When one considers the various processes which control the beat of cardiac tissue, the different metabolic requirements of these processes, the spatial relationships, and the cyclical nature of the metabolism, it is easy to understand why it is so difficult to correlate analyses for substances containing high-energy bonds and for other metabolic intermediates with the level of activity of the cardiac muscle.

Summary

1. The actions of thirteen metabolic substrates and intermediates on the rabbit auricle have been investigated, and it was found that they all depress the normal auricle, with regard to both rate and amplitude, except citrate which at higher concentrations stimulated the rate.

2. The behaviour of the beating auricles during depletion of substrate was observed and the abilities of various substrates to revive the auricles have been determined. It was found that only glucose and pyruvate produced marked effects; glucose produced a more marked effect on muscular relaxation, and pyruvate acted particularly upon the rate.

3. The actions of eight metabolic inhibitors on the rabbit auricle have been investigated and each inhibitor was found to produce a characteristic effect. The actions of these inhibitors on the functioning of the auricles does not always parallel their commonly accepted actions on enzymatic systems, and it is concluded that unknown pathways of metabolism exist in cardiac muscle.

4. The abilities of substrates to revive auricles that have been depressed by inhibitors was studied, and the bearing of these results on the specificity of inhibitor action and the nature of myocardial enzyme systems are discussed.

5. None of the observed actions of metabolic substrates and inhibitors are due to an action on the acetylcholine equilibrium in the auricular muscle, because the presence of atropine did not modify them.

6. The mechanisms by which the auricles can be stopped in diastole or systole are discussed in the light of the information obtained on the actions of substrates and inhibitors.

7. It is questioned whether changes in amplitude are directly related to contractile mechanisms. The results obtained could be best explained by assuming that the actions of the substances investigated were upon the mechanisms by which the propagated impulse stimulates the actomyosin to contract into its low-energy state. This important phase of the cardiac functioning is termed the "excitatory reaction." Depression of this excitatory reaction, either by reducing the excitatory state at the cellular membrane or by increasing the stimulation threshold of the contractile units, leads to a reduced number of units contracting and consequently to a reduced amplitude.

8. Changes in rate, amplitude, and degree of relaxation were shown to be relatively independent of each other. From the actions of substrates and inhibitors, as well as substrate depletion and anoxia, on the auricles, an attempt was made to correlate certain phases of metabolism with the mechanisms involved in rate, amplitude, and relaxation. It was concluded that contraction and relaxation are dependent on a certain supply of ATP but that the rhythmic discharge of impulses and the excitatory reaction are more directly dependent on the oxidative reactions of metabolism, particularly the tricarboxylic acid cycle.

9. Some general problems in the correlation of cardiac metabolism with cardiac function are discussed.

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ALLERGIC REACTIVITY TO SIMPLE ALIPHATIC ACIDS IN MAN*

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Data on allergic reactivity to simple aliphatic compounds are very scanty. Indeed the only substance of this kind known as a fairly frequent cause of contact dermatitis among workers in laboratories and in industry is formalin (1, 2, 3). There is one remarkable report on urticaria due to acrolein and other aliphatic aldehydes (including formaldehyde) (4). As to acetic acid, Kern (5) mentions that he had been told by physicians employed in plants manufacturing this acid of the occurrence of contact dermatitis among their workers.

Under these circumstances, and still more for reasons of the unique specificities of the reactions uncovered, it appears worthwhile to record the clinical experience and the result of subsequent testing of one of us (H. E. R.).

H. E. R., 22 years old, female, of British-German stock, suffered from hay fever from childhood until the age of 20 when she left her home state, New Mexico. Since then no signs of hay fever were noted.

In 1948, when handling animal cadavers preserved in formol, she experienced a rash on her hands and lower arms, tearing of the eyes and a generalized pruritus. In 1949, while working in a pathological laboratory, it was noted that formalin vapors caused violent lacrymation and pain in the nose, so that she had to give up all work which brought her into contact with formalin.

On July 20, 1950, she started work in this hospital. This work involved the pipetting of anhydrous acetic anhydride with a safety pipette. She was exposed to the vapors of this substance and probably was in direct contact with traces of the substance when handling the test tubes. After about 14 days of this exposure, small red lesions were observed on the wrists and forearms. This eruption repeatedly appeared immediately after handling acetic anhydride, and disappeared after a day or two. The eruption was accompanied by marked itching. Otherwise the personal history is non-contributory.

Family history: Two out of three brothers have hay fever. One of them also had had a rash on his face which was thought to be of allergic nature. No other history of allergic affections in the family can be recalled.

On the first examinaion on August 25, 1950, there were found on both forearms and the volar aspect of the hands, on the radial side of the right hand and ulnar half of the left, a number of reddish lesions. These were 1 to 3 mm. in size, often grouped, and sometimes vesicular in appearance. The lesions itched. The distribution on the hands was related to the areas which came in contact with the glassware containing acetic anhydride.

No other findings deviating from the normal were recorded. There was no dermographism or other indications of exaggerated reactivity of the skin.

Work was transferred to a hood, and the hands and arms were protected by rubber gloves and a long-sleeved gown. Under these protective measures the

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affection disappeared and did not come back, except once when for experimental purposes, the arms were deliberately left unprotected during one working period.

Testing: Repeated patch tests were made on the back with the aid of small pledgets of cotton saturated with aqueous solutions of the respective compounds and covered with "Elastopatch" (Duke Laboratories, Inc.). Readings were made after 24 hours and subsequently for several days. Similar patches applied to the skin of a non-sensitized person showed no reactions. All tests reported here were made "blind", that is, H. E. R. did not know the nature of the material applied to any given site. The results of the patch tests are presented in Table I.

The term "positive" employed in this table needs some amplification. In contrast to the usual experience in testing typical contact-type eczematous allergies, intense itching was reported on the positive sites within minutes after the application. After 24 hours the site was reddened and showed a few small vesicles. The reaction disappeared earlier than is usual, i.e. within 48 hours.

TABLE I

Patch tests

COMPOUND	CONCENTRATION MOLAR	REACTION
Acetic anhydride	1/2	Positive
Acetic anhydride	3/10	Negative
Acetic acid	1/2	Doubtful
Propionic anhydride	1/2	Negative
Sodium acetate	2/3	Negative
Sodium formate	2/3	Negative
Sodium proprionate	2/3	Negative
H_2O		Negative

Reddening was seen and itching reported at the sites tested with acetic acid and acetic anhydride, when work with acetic anhydride—which had been interrupted for a few days—was resumed on the seventh day after testing ("Flare-ups").

Impressed by the immediate reaction to exposure and in the patch tests, a series of intradermal tests was started.

For this purpose, volumes of 0.01 to 0.02 ml of aqueous solutions in the concentrations indicated in Table II were injected intradermally. Controls were performed by testing each agent in twice the concentrations indicated in Table II in three non-sensitized persons; four additional control subjects each received acetic and formic acid only. In the controls a sharp stinging sensation was noted with the acidic compounds and also at the site where the distilled water was injected, but this subsided within seconds. There was no visible reaction beyond the minimal traumatic one; the tiny wheal disappeared within less than 3 minutes; and there was no itching.

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The positive reactions in H. E. R. consisted of the large wheals, as noted in Table II, a flare of 2.5 cm. or more in diameter and definite itching. The reactions reached their maximum in about 15 minutes (when the readings given in Table II were taken) and were at the vanishing point after 30 to 40 minutes. There was outspoken itching. The negative sites showed none of these signs.

The intradermal tests were done repeatedly during the days from October 24 to October 26. When tests were repeated with the acids on November 15, reactivity had disappeared. It reappeared a few days after voluntary clinical reexposure to acetic acid.

TABLE II

Intradermal tests

COMPOUND	CONCENTRATION MOLAR	REACTION
Acetic anhydride	1/10	Wheal (10 mm) + Flare
Acetic anhydride	1/100	None
Acetic acid	1/5	Wheal (10 mm) + Flare
Acetic acid	1/10	Wheal (7 mm) + Flare
Acetic acid	1/50	None
Formic acid	1/5	Wheal (12 mm) + Flare
Propionic anhydride	1/10	Wheal (10 mm) + Flare
Butyric acid	1/5	None
Sodium acetate	1/5	None
Sodium formate	1/5	None
Sodium propionate	1/5	None
Formaldehyde	1/50	Wheal (8 mm) + Flare
Paraldehyde	1/5	None
Acetal	1/5	None
N-acetylglucosamine	1/5	None
Dist. water		None

Three months after the disappearance of the hypersensitivity phenomena described in the preceding paragraphs, H. E. R. was again intradermally injected with N/10 acetic anhydride and also with N/5 hydrochloric acid. No wheal and flare reaction or, for that matter, any other reactivity exceeding that described from control persons were noted. It would appear, that H. E. R. did not normally show an increased unspecific reactivity to acids in the sense as, for instance, described for alkali by Burckhardt (6) as a frequent occurrence in eczema caused by alkalis. This impression is further confirmed by the absence of exaggerated reactions to her frequent contacts with various acids in the course of her work.

Serum was obtained from H. E. R. on October 18 and two volunteers were injected on several sites with 0.05 ml of the undiluted serum. No indication of passive transfer was obtained when these persons were tested after 48 hours with formic acid and acetic acid.

DISCUSSION

From the clinical symptoms as well as the outcome of the skin testing it appears that the sensitization was of the immediate as well as eczematous type. There is both a family and a personal history of hay fever. Contact with formalin had caused in the past a sensitization apparently similar to the one described here with acetic acid.

Sensitization proved to be clinically of slight intensity.

As far as can be ascertained, this is the first study of sensitization to acetic acid.[1]

In addition to sensitivity to formaldehyde, dermal sensitivity of the immediate type was found to formic, acetic, and propionic acid—but not to butyric acid—or the respective anhydrides. Relatively high concentrations of the acids were required in order to elicit a dermal reaction: A M/10 solution of acetic anhydride represents a concentration of approximately 1 per cent, the molecular weight of this compound being 102. Whether this is correlated to the low degree of sensitization in this particular case, can of course not be decided from a single observation. It is of interest to note, that the threshold of dermal reactivity to formaldehyde—presumably a sign of previous sensitization—was appreciably higher than that to the acids.

Unfortunately, it was impossible to obtain information, whether one or more than one reagin was involved in this case. The latter possibility might have been considered in view of the two dermatitis-provoking exposures experienced during the past two years, namely to formalin and to acetic anhydride. The absence of transferable antibody obviated the performance of the crucial test of cross neutralization.

Of particular interest appears to us the observation that the sensitization to the acids did not involve reactivity to the corresponding sodium salts. It is pertinent to note that the (weak) aliphatic acids are but slightly dissociated, whereas the sodium salts are present in solution in the ionized form (7); and also that the acetate ion is very easily metabolized by living cells.

The methyl and acetyl radicals are known to influence the chemospecificity of proteins and to be important for the specificity and antigenicity of biological polysaccharides such as pneumococcal type I specific substances (see for references and discussion (8), pp. 51, 108, 174, and 213). Thus it was of interest to investigate whether an acetyl specificity of this kind would be involved in our case. We were able to test this point thanks to Dr. E. A. Kabat, from whom a sample of N-acetylglucosamine was obtained. The dermal reaction to this compound was repeatedly negative. We conclude that acetyl specificity is not

[1] The anhydrides employed in these experiments are practically instantaneously hydrolysed in aqueous solution to the corresponding acids.

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concerned here. Thus it would appear unlikely that the sensitizing property of the anhydride could be ascribed to its ability of acetylating protein (or other body substances).

Specificity to acetate (ion) or acyl radical being excluded, the only possible explanation of our observations appears then to be, that the specificity is directed to the *acid as such.*

The negative outcome of the test with paraldehyde and acetal further defines the specificity range of this case.

These observations in man were anticipated in a remarkable way by Jacobs et al. (9, 10) in experiments on the sensitization of guinea pigs by intracutaneous injection of various anhydrides. Among others, sensitization was reported with acetic, propionic, maleic, and n-caproic anhydrides. Dermal reactions of the wheal-and-erythema type were obtained in scratch tests with all of these anhydrides except propionic anhydride. With the latter compound, only reactions of the delayed type (with the patch test method) were obtained. No tests with the respective anions or acyl radicals were reported. These results emphasize once more the essential similarity of the reaction mechanisms to sensitization in man and in lower animals (see (11)).

SUMMARY

1. A case of sensitization to acetic anhydride is described. The reactions were of the eczematous and immediate type.

2. Dermal reactivity to acetic acid and acetic anhydride was not accompanied by reactivity to the anion nor to the acetyl radical. It would then appear that the specificity is directed to the acid as such.

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FATTY ACID METABOLISM

III. REACTIONS OF CARBOXYL-LABELED ACETIC ACID IN LIVER AND KIDNEY*

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Recent studies with isotopic octanoic (1) and butyric acids (2) revealed that their transformation to ketone bodies in liver occurs by condensation of a 2-carbon intermediary (presumably acetic acid or a derivative thereof) formed by successive β oxidation of the fatty acid. The importance of acetate in the intermediary metabolism of fats and proteins is further emphasized by the demonstration, by Bloch and Rittenberg (3, 4), that isotopic acetyl groups are formed in the breakdown of a variety of fatty acids and amino acids labeled with deuterium. Though acetate has been found to take part in such anabolic reactions as the formation of acetoacetate (5), cholesterol (6), and fatty acids (7) in animals, and succinic acid (8), butyl alcohol (9), and citric acid (10) in lower organisms, little is known concerning its oxidative catabolism.

Inasmuch as the immediate oxidation products of acetate, *e.g.* glycolic and glyoxylic acids, are metabolized slowly if at all in tissues in which acetate is rapidly broken down (11), these substances are unlikely to be intermediates of acetate oxidation in animal tissues. It is more probable, therefore, that condensation reactions precede its ultimate degradation to CO_2.

In continuation of our investigation of the metabolic pathways of fatty acids, we are making a study of the oxidation of acetate in animal tissues, utilizing the stable carbon isotope, C^{13}, incorporated in the carboxyl group. The object of the present study was to elucidate the mechanism of the condensation of acetate to ketone bodies in liver, and to obtain information concerning the possible intermediate stages of acetate oxidation in kidney, which proceeds without the apparent formation of ketone bodies.

EXPERIMENTAL

Preparation of Labeled Acetic Acid.—Acetic acid, labeled in the carboxyl group, was prepared by the action of methyl sulfate on 0.98 gm. (0.02 mole) of isotopic sodium cyanide according to the method of Walden (12) and subsequent hydrolysis of the acetonitrile by aqueous alkali. The

* With the technical assistance of Mary Cammaroti, Lafayette Noda, and Ruth Wilson.

product was isolated as the sodium salt and its purity checked by determination of the Duclaux constants. The sodium cyanide contained 1.30 atoms per cent C^{13} and the sodium acetate contained 2.16 per cent, equivalent to a C^{13} content of 4.32 per cent in the carboxyl carbon atom.[1] The yield was 13.75 mm or 83 per cent based on the cyanide.

Incubation with Liver Slices and Recovery of Products.— Approximately 1 mm of the labeled sodium acetate was added to 100 ml. of the buffer solution previously described (1) and a 30 ml. aliquot removed for acetate assay and determination of the Duclaux constants. 30 ml. aliquots were added to each of two 125 ml. Warburg type flasks, each containing 1.25 gm. of liver slices in the main compartment, alkali-soaked filter paper in the central well, and 2 N sulfuric acid in the side cup. All transfers were carried out in a nitrogen atmosphere to avoid contamination with atmospheric CO_2. The flasks were shaken in oxygen for 2 hours at 37.5°; the oxygen consumption was measured by a mercury-filled gas burette, attached to the two flasks by a T-connection. The acid was then introduced from the side cup and shaking continued 15 minutes to release the respiratory CO_2. The alkali-soaked papers were removed and treated as previously described for estimation and collection of the respiratory CO_2. The medium was separated from the tissue, combined, and divided in two portions. One portion was steam-distilled according to the procedure of Van Slyke and Folch and the distillate made alkaline and redistilled. The second distillate, thus freed of non-volatile substances and volatile acids, contained the acetone derived by decarboxylation of acetoacetate during distillation. It was isolated as the Deniges complex according to the procedure of Van Slyke (14). The residue of the second distillation, containing the sodium salts of the volatile acids, was acidified, distilled, and the Duclaux constants determined. It was finally redistilled and the acids neutralized, phenolphthalein being used as an outside indicator. The resultant solution of the sodium salts was then evaporated to dryness.

The second portion of the medium was treated by the Van Slyke (14) procedure, previously described (1) for decarboxylation of acetoacetic acid and isolation of the acetone and CO_2, after which the hydroxybutyrate was oxidized with dichromate and the acetone isolated.

The tissue was then divided in two portions, one of which was steam-distilled to recover any volatile acid which might have been bound by the tissue. The amounts so adsorbed were insignificant, however. The second portion of tissue was extracted successively with alcohol and ether, thus yielding a tissue non-lipid and lipid-free fraction.

In the experiments with kidney slices the procedure was the same except that the isolation of ketone body fractions was omitted, since trial

[1] The C^{13} content is expressed as atoms per cent (C^{13} excess, calculated from the equation: atoms per cent C^{13} = (moles C^{13} × 100) / (moles C^{12} + moles C^{13}) = 1.1

experiments with normal acetic acid showed no trace of these substances. The non-volatile residue after distillation of the unutilized acetate was found to contain excess C^{13}. To purify this fraction further it was extracted continuously for 48 hours with ether. By this process all of the excess C^{13} was extracted, but since the amount of extract was only sufficient for a C^{13} determination, further identification was impossible.

TABLE I

Distribution of Excess C^{13} in Products of Incubation of C^{13}-Labeled Acetic Acid with Liver and Kidney Slices from Fasted Rats

	Liver				Kidney			
	Experiment 1, 2.50 gm. tissue		Experiment 2, 2.50 gm. tissue		Experiment 1, 2.00 gm. tissue		Experiment 2, 1.53 gm. tissue	
		C^{13} excess		C^{13} excess		C^{13} excess		C^{13} excess
	mm	*atoms per cent*	*mm*	*atoms per cent*	*mm*	*atoms per cent*	*mm*	*atoms per cent*
Acetic acid, start	0.634	2.16	0.638	2.16	0.628	2.16	0.632	2.16
" " recovered	0.420	1.96	0.432	2.06	0.490	1.91	0.490	2.07
" " utilized	0.214		0.206		0.120		0.142	
O_2 consumed	0.494		0.550		0.388		0.537	
CO_2 evolved in respiration	0.514	0.92	0.574	0.95	0.269	1.06	0.521	0.99
Acetoacetic acid	0.0750		0.0638		0		0	
β-Carbon, direct		1.47		1.71				
" by distillation		1.62		1.71				
Carboxyl carbon		1.89		2.25				
β-Hydroxybutyric acid	0.0361		0.0277		0		0	
β-Carbon		0.72		1.41				
Non-volatile carbon					1.13	0.05	0.89	0.10
					mg.		*mg.*	
" " ether extract					8	0.18	4	0.28
Tissue lipids		0.03		0.01				0.01
" non-lipids		0.02		0.02				0.03

For the C^{13} determinations the solid fractions were converted to CO_2 by the Van Slyke and Folch wet combustion procedure (15). The fractions in solution were oxidized by a micro modification of the persulfate method of Osburn and Werkman (16).

Results

Ketone Body Formation in Liver.— In Experiment 1, the acetone, obtained as the Deniges complex formed *in situ* by the breakdown of acetoacetate in the presence of mercuric sulfate, contained 0.49 per cent C^{13}. This represents a C^{13} content in the carbonyl carbon of 3 × 0.49 = 1.47 per cent (Table I). As a check on the specificity of this procedure, and to

avoid the possibility of inclusion of other carbon-containing substances, the precipitation with mercuric sulfate was employed on another portion of acetone, freed, by distillation, from non-volatile substances and volatile acids. This sample had a slightly higher C^{13} content, 0.54 per cent, representing 1.62 per cent in the carbonyl carbon. In Experiment 2, the C^{13} contents of the two acetone fractions were equal, 0.57 per cent, representing an excess of 1.71 per cent in the carbonyl carbon.

In both experiments the carboxyl carbon atoms had a somewhat higher C^{13} content than the corresponding β-carbon atoms. To verify this observation we carried out a third experiment (not included in Table I) with a new sample of labeled acetate containing 8.32 per cent C^{13} in the carboxyl group. In this experiment part of the acetoacetate was broken down by the procedure described and another portion was decarboxylated with aniline citrate according to the method of Edson (17). Since the latter method does not require heating and is highly specific for β-keto acids, it is probably more reliable than heat decarboxylation. By this procedure we also found a somewhat greater C^{13} content in the carboxyl carbon than in the β-carbon, the respective values being 2.58 and 2.22 per cent. The results of this third experiment are somewhat ambiguous, however, in that the CO_2 from heat decarboxylation had a C^{13} content of only 1.70 per cent.

The acetone derived by oxidation of β-hydroxybutyrate also contained excess C^{13}, a result to be anticipated considering the close metabolic relationship between acetoacetate and hydroxybutyrate. However, the calculated C^{13} content of the β-carbon atom of hydroxybutyrate was lower in each experiment than the corresponding value for acetoacetate. The quantitative significance of these differences is difficult to assess, in view of the possibilities of contamination by acetone or other substances arising by oxidation of substances other than hydroxybutyric acid.[2]

The C^{13} content of the carboxyl carbon of hydroxybutyrate could not be determined, since in the oxidation of hydroxybutyrate to acetoacetate the carboxyl CO_2 was diluted by relatively large quantities of CO_2 derived from other readily oxidizable substances present in the medium.

Complete Oxidation of Acetic Acid in Liver—In Experiment 1 the 0.314 mM of CO_2 evolved by respiration contained 0.92 per cent C^{13}. Since the labeled acetic acid had a C^{13} content of 2.16 per cent, 0.92 × 100/2.16 or 42.5 per cent of the respiratory CO_2 was derived by oxidation of isotopic acetate. This is equivalent to 0.314 × 0.425/2 = 0.067 mM of acetate or 0.067 × 100/0.214 = 31 per cent of the amount utilized. In Experiment 2, similarly calculated, 44 per cent of the respiratory CO_2 came from the substrate, representing the complete oxidation of 0.082 mM or 40 per cent of the utilized acetate.

[2] In a third experiment the C^{13} contents of the acetone derived from acetoacetate and hydroxybutyrate were about equal, the respective values being 0.71 and 0.77 per cent.

Comparison of the Duclaux constants of the acid before and after incubation reveals no significant formation of other volatile acids in the incubation procedure (Table II). However, a slight decrease in the C^{13} content of the unutilized acetate was observed, which seems to indicate a slight dilution by normal acetate formed during incubation. The quantity of preformed acetate in liver is negligible.

Acetate Oxidation in Kidney—In Experiment 1, 0.129 mM of acetate was utilized and 0.299 mM of CO_2 was obtained, with a C^{13} content of 1.06 per cent. Hence, 1.06 × 100/2.16 or 49.1 per cent of the total CO_2 came from the substrate, representing the complete oxidation of 0.299 × 0.491/2 = 0.077 mM or 0.077 × 100/0.129 = 59 per cent of the utilized acetate.

TABLE II

Duclaux Constants of Volatile Acid Fractions

	Per cent of total volume distilled						
	12.5	25	37.5	50	62.5	75	87.5
	Per cent of total acidity in distillate						
Normal acetic acid	11.8	23.9	36.8	50.5	65.4	81.2	95.6
Isotopic " "	12.0	24.4	38.1	49.0	63.7	79.2	93.9
After incubation, liver	14.7	26.3	38.2	50.3	64.8	79.8	93.8
" kidney	12.4	23.9	36.8	50.3	65.4	80.8	94.5

In Experiment 2, similar calculation reveals that 83 per cent of the utilized acetate was completely oxidized.

After removal of the acetate by distillation the non-volatile residue, containing 1.13 and 0.89 mM of carbon in the respective experiments, had a small C^{13} excess, all the excess C^{13} of which was extractable with ether. The amount of this fraction was so small, however, that only sufficient was available for a C^{13} determination. Consequently further identification was impossible.

As in the liver experiments, the Duclaux constants reveal that the recovered acetic acid was unaccompanied by other volatile acids, but there was similarly observed a small dilution by normal acetic acid, due either to acetate formation from tissue constituents during incubation or to preformed acetate.

Recovery of C^{13}—As shown in Table III, the respiratory CO_2 and ketone bodies account for 63 and 84 per cent of the C^{13} utilized as acetate in the

respective liver experiments. The C^{13} content of the non-volatile fractions was not low for an accurate estimation, but could not have represented more than 5 per cent of the total. The remainder was probably incorporated in the tissue by synthetic reactions. Although the tissue fractions did not contain significant C^{13} excesses, this does not signify that none of the excess carbon for C^{13} was incorporated in the tissue, as the small quantities involved would be masked by the relatively large mass of normal tissue carbon.

In the respective kidney experiments the respiratory CO_2 and the non-volatile fraction account for 70.5 and 96.7 per cent of the total utilized C^{13}.

TABLE III

Percentage of C^{13} Utilized As Acetic Acid, Appearing in Various Fractions

	Liver		Kidney	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Acetoacetic acid	26.3	55.5	0	0
Lactic acid	5.6	8.7	0	0
Respiratory CO_2	31.2	39.5	59.8	82.5
Non-volatile substances	*	*	10.7	14.2
Urea	0	0	0	0
Total accounted for	63.1	83.7	70.5	96.7

* Not determined.

DISCUSSION

The equal distribution of excess isotope between the carbonyl and carboxyl carbon atoms of acetoacetic acid formed from isotopic octanoate indicates that the metabolism of fatty acids in liver proceeds through the intermediary formation of a 2-carbon substance. The similar results reported herein with carboxyl-labeled acetic acid may be taken as additional confirmation of this postulate and further emphasizes the close relationship between acetic acid and this 2-carbon intermediary.

Of the total ketone bodies formed, only a part was derived from the acetic acid. This is indicated by comparison of the C^{13} contents of the respective substances. In Experiment 1, for example, the acetoacetate contained (1.62 + 1.39) 3 = 0.88 per cent C^{13}, whereas the acetic acid had a C^{13} content of 2.16 per cent. If we assume that this difference represents dilution of the isotopic acetoacetate by non-isotopic acetoacetate derived from tissue constituents, we may calculate that 0.88 × 100/2.16 = 41 per cent of the total acetoacetate came from the

substrate and 59 per cent from the tissue. Similar calculations for Experiment 2 indicate that 45 and 55 per cent were derived from the substrate and tissue, respectively. Though the origin of this non-isotopic acetoacetate is uncertain, the most probable source is the fatty acids of the liver lipids. If these fatty acids were broken down to acetic acid in the course of their conversion to ketone bodies, a dilution of the isotopic acetic acid would be expected.

Although a dilution was observed, it was not as large as expected. In Experiment 1, for example, 60 per cent of the 0.111 mM of ketone bodies came from the tissue. It should have required the production of 0.111 × 0.60 × 2 or at least 0.133 mM of acetic acid. Since 0.634 mM of acetate was originally present, the minimum expected dilution would be 0.133 × 100/(0.634 + 0.133) = 17 per cent, whereas the observed dilution was 9 per cent. In Experiment 2, the minimum dilution should again have been about 17 per cent, whereas the observed dilution was 11 per cent.

It should be pointed out that the expected dilution would be observed only if equilibrium exists between the biologically formed acetic acid and the isotopic substrate. If the rate of condensation is rapid in comparison with the diffusion, it is conceivable that the "biological" acetic acid, being produced intracellularly, is preferentially condensed before mixing with the major portion of extracellular isotopic acetate. This would be true, particularly, if the biological 2-carbon intermediary is not acetic acid itself, but a phosphorylated derivative such as acetyl phosphate, which would not be expected to diffuse readily.

Two other observations point to the possibility that the biological intermediary is not acetic acid itself. First, the condensation of acetate does not occur in the absence of oxygen.[3] Under anaerobic conditions no acetate is utilized and no ketone bodies are formed. Since oxidation is not involved in the condensation of acetate to acetoacetate, it is conceivable that oxidative energy is required to convert acetate to an active form which can undergo the condensation. Lipmann (18) has brought forward evidence to show that the "active" acetate concerned in the many fold reactions of pyruvic acid is acetyl phosphate. This substance may be involved here also.

The second observation is the apparently greater concentration of C^{13} in the carboxyl than in the carbonyl group. This is difficult to explain on the basis of a condensation of identical molecules. If we assume, however, that the substrate molecules react, not only with one another, but also with a more active biological 2-carbon compound, the higher activity of the latter might be manifested by a greater tendency to act as the acetylating

[3] Unpublished observation.

agent in the condensation. With acetyl phosphate as an example, the reaction could be formulated as follows:

$$CH_3CO(O)PO_3H)^- + CH_3C^{13}OOH \rightarrow CH_3COCH_2C^{13}OOH + (H_2PO_4)^-$$

Other Oxidation Pathways of Acetic Acid in Liver—The C^{13} content of the respiratory CO_2 represents the complete oxidation of 30 to 40 per cent of the utilized isotopic acetate. Inasmuch as acetoacetate is appreciably oxidized by fasted rat liver (19), part at least of the isotopic CO_2 of respiration represents the breakdown of acetoacetate.

The question remains whether all of the isotopic CO_2 represents breakdown of ketone bodies or whether acetate is metabolized by some other pathway. If all of the acetate goes through ketone bodies, the C^{13} content of the respiratory CO_2 would be equal to or lower than the C^{13} content of the ketone bodies. If the C^{13} content of the respiratory CO_2 were significantly higher than the ketone bodies, this would constitute proof for another oxidation pathway. In Experiment 1 the acetoacetate contained 0.88 per cent C^{13}, whereas the respiratory CO_2 had 0.92 per cent; in Experiment 2 the respective values were 0.99 and 0.95 per cent; in a third experiment they were 1.20 and 1.21 per cent. Thus, in all three experiments the isotopic contents were approximately equal. Inasmuch as we are reluctant to place too great reliance on small quantitative differences in view of the possibility of unknown sources of error in the C^{13} determinations, the above data are regarded as inconclusive as to whether or not acetate is oxidized through other pathways than acetoacetate. However, the approximate equality of the C^{13} contents of the acetoacetate and the CO_2 suggests that practically all of the substances involved, whether from the substrate or derived from it, pass through the ketone body stage prior to complete oxidation.

Acetate Oxidation in Kidney—The results of these experiments gave no conclusive information concerning the possible intermediates of acetate oxidation in kidney. The presence of C^{13} in the non-volatile material extractable with ether does suggest that possibly C_4-dicarboxylic acids or other components of the Krebs cycle may be involved. These possibilities are now being studied.

SUMMARY

1. The distribution of C^{13} in acetoacetic acid formed by condensation of acetic acid, labeled with C^{13} in the carboxyl group, indicates that the condensation proceeds by coupling of two acetyl groups.

2. Of the total acetoacetate, 41 to 45 per cent came from the isotopic acetate and the remainder presumably from constituents of the liver tissue.

3. Although the results establish the ketone bodies as an intermediate

stage of acetate metabolism in liver, the data are inconclusive as to whether or not acetate is also metabolized by some other pathway not involving ketone body formation.

4. A decrease in the C^{13} content of the acetate recovered after incubation demonstrates the formation of acetate from tissue constituents.

5. Acetic acid is oxidized in kidney without the appreciable accumulation of intermediates. The presence of a small quantity of isotope in the non-volatile fraction extractable with ether suggests that the C_4-dicarboxylic acids or other components of the Krebs cycle may be intermediates of acetate oxidation in kidney.

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Glycolytic Control Mechanisms

I. INHIBITION OF GLYCOLYSIS BY ACETATE AND PYRUVATE IN THE ISOLATED, PERFUSED RAT HEART*

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Earlier studies with the perfused rat heart have shown that the rate of glycolysis was inhibited by the addition of fatty acids or ketone bodies to the perfusion medium (1–4). The rate of glucose phosphorylation was shown to be an important factor determining the over-all rate of glucose uptake by the heart when membrane transport was stimulated by insulin. On the basis of changes in the tissue content of glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-diphosphate in the presence and absence of fatty acids, ketone bodies, or pyruvate, Newsholme, Randle, and Manchester (2) proposed that phosphofructokinase was the rate-limiting step of glycolysis in the perfused rat heart, and that the activity of phosphofructokinase was decreased during the oxidation of these metabolic substrates. Decreased phosphofructokinase activity resulted in increased levels of glucose-6-P, and it was further proposed that hexokinase, being product-inhibited, was limited by the accumulation of glucose-6-P. Reports from several laboratories (5–8) have since suggested that citrate may have an important role in the regulation of glycolysis, as indicated by the ability of citrate to inhibit phosphofructokinase in the cell-free system, and its accumulation in cardiac muscle under conditions of low carbohydrate utilization (6–8). Variations in the citrate content of a tissue such as the heart, which maintains a fairly constant respiratory activity under conditions of constant work, is of great interest in terms of the over-all regulation of the citric acid cycle, and is a problem which merits further attention.

In this investigation, isotopic glucose was used to determine the major pathways of glucose metabolism in the presence and absence of insulin, after the addition of either acetate or pyruvate to the perfusion medium. These substrates were chosen as suitable fuels to compete with glucose for respiration, since they have previously been shown to be readily metabolized by the perfused rat heart (9, 10). The levels of most of the glycolytic intermediates, the adenine and pyridine nucleotides, and several of the citric acid cycle intermediates and related amino acids have been measured after the addition of either acetate or pyruvate to hearts perfused with glucose, in order to gain further insight into the mechanisms involved in the control of glycolytic activity. The mass action ratios of the glycolytic reactions, calculated on the basis of the observed intermediate levels, showed that the steps mediated by hexokinase, phosphofructokinase, pyruvic kinase, and probably glyceraldehyde-P dehydrogenase are far displaced from equilibrium, whereas phosphoglucose isomerase, phosphoglycerate mutase, enolase, lactic dehydrogenase, α-glycerophosphate dehydrogenase, and probably P-glyceric kinase are normally close to equilibrium, with aldolase and triose-P isomerase occupying intermediate positions. Enzymic steps far removed from equilibrium are potential sites of metabolic control. Phosphofructokinase was identified as the enzyme controlling glycolytic flux after the addition of acetate, and inhibition of glycolysis was associated with increased levels of citrate. After the addition of pyruvate, citrate also accumulated, but inhibition at other sites, tentatively identified at the glyceraldehyde-P dehydrogenase and pyruvic kinase steps, had a marked effect on the distribution pattern of the glycolytic intermediates, and modified the dominant inhibitory effect of citrate on phosphofructokinase. A preliminary report of part of this work has been published (11).

EXPERIMENTAL PROCEDURE

Animals and Perfusion Technique—Male albino rats of Sprague-Dawley or Wistar strain, weighing 220 to 280 g, fed on stock Purina chow, were anesthetized with a 50% O_2-50% CO_2 gas mixture (12). The heart perfusion technique was similar to that described previously (1, 12), and all hearts were perfused for 15 minutes in a separate perfusion apparatus with oxygenated Krebs-bicarbonate medium containing 5 mM glucose. This procedure served to wash out hormones and produce a metabolically stable preparation (12–14).

Fluorescence Studies on Isolated Rat Heart—Changes in the state of oxidation-reduction of the pyridine nucleotides near the surface of the isolated beating heart were measured by means of a microfluorometer similar to that described by Chance *et al.* (15, 16). The perfusion apparatus was modified so that perfusate equilibrated either with 95% O_2-5% CO_2 or with 95% N_2-5% CO_2 could pass into the cannulated aorta by turning a 3-way stopcock. The heart was placed in a water-jacketed chamber made of Pyrex glass, and a horizontal beam of excitation light (366 mμ) was focused on an area of ventricle approximately 2 mm². The depth of the field of observation which gave half-maximal fluorescence emission signal was 0.25 mm. Changes in the fluorescence intensity from the initial aerobic steady state were proportional to changes in the sum of the total reduced

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pyridine nucleotides found in the heart, as determined by analysis of tissue extracts in a carefully controlled series of experiments (Fig. 1). Increases in the proportion of reduced pyridine nucleotides in the heart were achieved by the addition of Amytal (0.5 to 2.0 mM) to the perfusate. The changes in the tissue content of NADPH were small compared with those of NADH. The calibration shown in Fig. 1 may not be used to obtain the absolute tissue levels of reduced pyridine nucleotides in other experiments, however, owing to changes in the high fluorescence blank of the heart chamber and the sensitivity of the detecting system. This technique for the measurement of the fluorescence intensity of the perfused heart was of particular value in determining the kinetics and net directional changes in the levels of reduced pyridine nucleotides, and fluorescence intensity changes relative to the aerobic-anaerobic transition have been found to be relatively consistent with different hearts.

Tissue Oxygen Tension—This was measured by using a gold wire, 50 μ in diameter, as the cathode. The wire was insulated with epoxy resin except for the exposed tip. The technique for the preparation and calibration of such flexible electrodes has been previously described (17, 18). The electrode was inserted into the ventricular muscle through a small hole so that the tip was situated a little distance away from the damaged tissue and about 0.5 mm below the surface. A silver-silver chloride wire immersed in the perfusate served as the anode, and the polarizing voltage was −0.6 volt.

Isotope Techniques—For studies with radioactive glucose, hearts were perfused with medium containing 5 mM glucose, 10 μC/100 ml of ^{14}C-glucose (uniformly labeled) and, when present, 10 mM sodium acetate, 10 mM sodium pyruvate, or 2 milliunits per ml of insulin. Duplicate samples of perfusate were removed for chemical analyses and counting initially, and after 60 minutes of perfusion. The method for the collection of the metabolic $^{14}CO_2$ and the counting techniques were the same as those described previously (12). Counts were converted to micromoles of glucose equivalents by dividing by the specific activity of glucose, which was found not to change during perfusion. Results recorded as medium count decrease represent the difference between counts removed from the medium as ^{14}C-glucose and those returned as ^{14}C-lactate and other ^{14}C products of glucose metabolism.

At the end of the perfusion, the heart was flushed through with 4 ml of cold, nonradioactive medium to remove radioactive perfusate from the capillary bed and most of the extracellular space, blotted, and divided into segments for dry weight and glycogen determinations. Glycogen was precipitated by the method of Good, Kramer, and Somogyi (19), washed three times with 95% ethanol, and hydrolyzed with 2 N H_2SO_4. An aliquot of the neutralized hydrolysate was counted to obtain the incorporation of ^{14}C-glucose into polysaccharide, while an aliquot of the ethanolic KOH supernatant of the heart digest was counted to obtain the total counts remaining in the heart. These counts represent the conversion of ^{14}C-glucose to unidentified ^{14}C intermediates which remain in the heart.

The extracellular space was determined in separate experiments by means of ^{14}C-sorbitol, uniformly labeled (20). Hearts were perfused for 15 minutes with medium containing 5 mM glucose and 0.5 g of unlabeled sorbitol per liter, transferred to recirculation circuits, and perfused with 15 or 20 ml of a similar medium containing 2.5 μC/100 ml of ^{14}C-sorbitol. The volume of the sorbitol space was estimated from the ratio of counts in

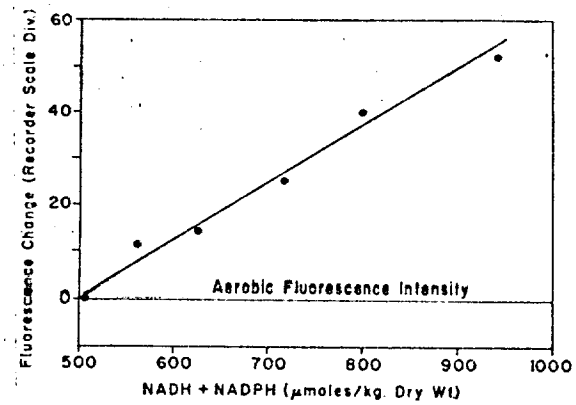

FIG. 1. Relationship between the fluorescence intensity of the perfused rat heart (480 mμ peak emission) and the content of reduced pyridine nucleotides. Hearts were perfused with 5 mM glucose under standard conditions. Reduction of pyridine nucleotides was achieved by the addition of Amytal to the perfusate, and the hearts were rapidly frozen when the fluorescence increase reached a constant level (2 to 3 minutes). The content of reduced pyridine nucleotides in the hearts at the moment of freezing was determined by tissue analyses.

the perchloric acid heart extract to those in the perfusate. The volume of the intracellular water was calculated as the difference between the total water content and the volume of the sorbitol space (20).

Preparation of Perchloric Acid Heart Extracts—At the end of perfusion, hearts were quickly frozen by means of tongs cooled in liquid nitrogen (21), and the frozen tissue was powdered in a stainless steel percussion mortar embedded in Dry-Ice. Approximately 150 mg of powder were weighed, dried to constant weight at 105°, and reweighed to obtain the water content of the tissue. A second aliquot (300 to 600 mg) was weighed while frozen, deproteinized by homogenizing in 0.6 N $HClO_4$ with Kontes glass homogenizing tubes, and centrifuged at 15,000 × *g* for 15 minutes. Great care was taken to avoid thawing of the powder during acidification. A second extraction of the protein pellet with perchloric acid was found to be unnecessary. A measured volume of supernatant was neutralized to pH 6 with 3 N K_2CO_3, and the precipitated potassium perchlorate was removed by centrifugation in the cold. The extracts were kept frozen between analyses, and the least stable intermediates, such as pyruvate, P-enolpyruvate, oxaloacetate, and NADP, were analyzed in fresh extracts. Analysis of extracts for hexose mono- and diphosphates, dihydroxyacetone-P, and ATP on successive days showed that these intermediates were stable to degradation at least over a 4-day period.

Preparation of Alkaline Heart Extracts—An aliquot of the frozen powder (200 to 300 mg) was mixed with 1 ml of 1.5 N ethanolic KOH (equal volumes of water and ethanol) while still cold, and heated with mixing for 60 seconds at 55°. The clear digest was cooled, and 1 ml of cold 0.5 M triethanolamine hydrochloride, pH 6.5, was added slowly with mixing. The extracts were carefully neutralized to pH 8 with 2 N HCl during vigorous mixing and centrifuged at 20,000 × *g* for 20 minutes in the cold. These extracts were used for the assay of NADH and NADPH.

Analytical Methods—The perfusate was analyzed for glucose (22), lactate (23), and glycerol (24). In some experiments, lipid was extracted from the heart by the method of Folch and Stanley (25). The metabolic intermediates were all measured

enzymically by coupling the reactions to appropriate enzymes involving oxidation or reduction of di- or triphosphopyridine nucleotides. A Beckman model DU or a Zeiss spectrophotometer was used for some of the assays, which were made in quartz cuvettes with a total fluid volume of 2.5 ml. Calculations were based on a molar extinction coefficient of 6.22×10^3 for NADH or NADPH at 340 mμ for a 1-cm light path. When greater sensitivity was desired, due to the low concentration of the intermediate in the heart, or in order to conserve the extract volume, a modified Eppendorf recording fluorometer (26) was used, with 1-cm quartz cuvettes containing 2.5 ml of reaction medium. At the highest useful sensitivity of the instrument, a fluorescence change of 5% full scale on the recorder corresponded to an NADH concentration of 10^{-11} mole in the cuvette, and represented the lowest concentration of an intermediate that could be detected. The normal sensitivity setting of the fluorometer was full scale deflection for 1.5 mμmoles of NADH. Assays with the fluorometric technique were performed individually, and calibration was achieved by means of appropriate intermediates added to each cuvette as internal standards after the initial reaction had reached completion. Most of the assays were run in duplicate, and reproducibility varied from 2 to 10% depending on the assay. The enzymes used for the assays were carefully checked for fluorescence blanks, and for contamination by other enzymes or substrates which would interfere with the accuracy or specificity of the reactions and the appropriate corrections made.

Metabolic intermediates present in the tissue extracts were analyzed by modifications of methods described in the literature (26–29). ATP, creatine, creatine-P, glutamate, aspartate, glutamine, and asparagine were present in high enough concentrations to permit accurate analyses in the Zeiss spectrophotometer with sample volumes of 0.1 to 0.2 ml. With the exception of the data presented in Table IV, all the other analyses were made with the fluorometric enzyme assay technique. In general, excessive amounts of cofactors or substrates necessary for the reactions were avoided, and except for the glutamate assay, which required 60 minutes for completion, the enzyme concentrations used were the minimum required for the reactions to reach completion in 2 to 5 minutes.

Citrate was determined with aconitase (27), which was isolated and purified up to the first ammonium sulfate fractionation step by the method of Morrison (30). A 0.1 M triethanolamine buffer, pH 7.4, was used for the assay of NADP, NADH, NADPH, glucose-6-P, fructose-6-P, fructose-1,6-di-P, and triose-P. The imidazole buffer recommended by Lowry *et al.* (29) for the determination of fructose-1,6-di-P was found to offer no advantages over the triethanolamine buffer. NADH and NADPH were measured in the same cuvette, which contained 0.1 mM pyruvate, 0.1 mM α-ketoglutarate, and 0.1 mM ammonium chloride as substrates, by the addition of lactic dehydrogenase followed by glutamic dehydrogenase when the first reaction utilizing NADH had reached completion. A buffer containing 50 mM triethanolamine buffer, 10 mM $MgCl_2$, and 5 mM EDTA (pH 7.0), was used for the determination of ADP and AMP. For this assay, it was essential to free the NADH from contaminating AMP (29). Glucose and ATP in the heart extract were determined by means of hexokinase and glucose-6-P dehydrogenase (27). The glucose oxidase method (22) was found to be unsuitable for analysis of glucose in the extract, owing to partial hydrolysis of glycogen by contaminating enzymes in the glucose oxidase. An alkaline buffer of 0.2 M glycin and 0.4 M hydrazine hydrate, pH 9.5, was used for the assay NAD, glutamate, α-glycerophosphate, and malate, the latt two intermediates being measured in the same cuvette.

Measurement of 3-P-glycerate and 2-P-glycerate by enzymi reactions coupled either to lactic dehydrogenase or to glyceraldehyde-P dehydrogenase (27, 29) yielded a fairly constant discrepancy between values obtained by the two methods. Th discrepancy can be ascribed to accompanying phosphatase activity in commercially available P-glycerate mutase preparatio causing hydrolysis of 2,3-di-P-glycerate to 3-P-glycerate (29 Consequently, addition of P-glycerate mutase after pyruvi kinase and enolase to the reaction mixture coupled to lact dehydrogenase yielded values for the sum of 3-P-glycerate an 2,3-di-P-glycerate, while addition of P-glycerate mutase aft P-glycerate kinase to the reaction mixture coupled to glyceraldehyde-P dehydrogenase gave values for the sum of 2-P-glycerat and 2,3-di-P-glycerate. Normally, 3-P-glycerate was measur by means of reactions coupled to glyceraldehyde-3-P dehydrogenase and 2-P-glycerate by reactions coupled to lactic deh drogenase.

All the enzymes, intermediates, and cofactors were obtain from Boehringer and Sons through the California Corporati for Biochemical Research, or from Sigma Chemical Compan The buffers used in the fluorometric assays were filtered throu fritted glass filters of fine porosity to remove dust and oth fluorescent particles. NAD and NADP were made up in wa and added directly to the cuvettes immediately prior to the ass NADH, made up in 0.1 M triethanolamine buffer, pH 8.0, w used similarly. Substrates were stored frozen for short perio as stock solutions and were added directly to the cuvette. T recovery of known amounts of glucose, ATP, glucose-6- fructose-1,6-di-P, NAD, and NADPH added to extracts rang from 94 to 100%. The recovery of NADH and NADPH vari from 75 to 95% in different experiments.

RESULTS

Metabolism of ^{14}C-Glucose—The effects of acetate and pyruv on the uptake and utilization of ^{14}C-glucose (uniformly label by the perfused rat heart are shown in Table I. In the abse of insulin, glucose uptake was 77 μmoles per g (dry weight) hour, and was decreased significantly by pyruvate ($0.05 > p$ 0.02) but not by acetate ($p > 0.1$). Lactate formation by cont hearts represented 9% of the glucose uptake, and was increa 3-fold by the addition of acetate. Lactate formation in t presence of pyruvate was high, since lactate was produced fr pyruvate as well as from glucose (10). The count decrease the medium, which represents the difference between cou removed from the medium as glucose and those returned pr cipally as ^{14}C-lactate, was decreased 50% by both acetate a pyruvate, indicating that lactate formation from glucose w similar in the presence of either substrate. With control hea formation of $^{14}CO_2$ accounted for 61% of the medium co decrease over the 60 minutes of perfusion, and incorporation glucose counts into glycogen for only 3%. Most of the rema ing counts were recovered in the ethanolic KOH supernatant the heart digest after glycogen precipitation, and represent conversion of glucose to metabolic products which remained the tissue. These compounds were not identified, but repres a surprisingly large fraction of the glucose uptake in confirmat of similar findings in other tissues (31). Their accumulation

TABLE I

Effects of acetate and pyruvate on uniformly labeled ^{14}C-glucose metabolism in perfused rat heart

Hearts after 15 minutes of prior perfusion with 5 mM glucose were transferred to recirculation circuits for further perfusion. Samples of perfusate were removed for analysis and counting initially, and after 60 minutes of perfusion. Net glycogen change is the difference between the mean glycogen content of hearts at the beginning and after 60 minutes of perfusion. Values shown are mean metabolic changes ± the standard error of the mean.

Additions to 5 mM glucose	No. of hearts	Glucose uptake	Lactate formation	Medium count decrease	Glucose to $^{14}CO_2$	Glucose to ^{14}C intermediates in heart	Glucose to ^{14}C-glycogen	Net glycogen change	Recovery of isotope
		μmoles/g (dry wt)/hr							%
No insulin									
Control	10	77 ± 7	14 ± 3	67 ± 4	41 ± 3	15 ± 1.2	2.2 ± 0.5	−4 ± 7	87
Acetate, 10 mM	6	61 ± 6	46 ± 4	36 ± 5	6.4 ± 1.0	15 ± 1.5	12.5 ± 2.4	+27 ± 7	94
Pyruvate, 10 mM	6	51 ± 4	223 ± 17	34 ± 2	6.5 ± 0.9	12 ± 1.2	14.4 ± 1.4	+27 ± 6	97
Insulin, 2 milliunits per ml									
Control	10	334 ± 12	147 ± 7	227 ± 10	96 ± 7	57 ± 1.5	46 ± 4	+59 ± 6	88
Acetate, 10 mM	10	179 ± 14	82 ± 12	132 ± 8	10.3 ± 0.8	33 ± 0.8	85 ± 7	+80 ± 8	97
Pyruvate, 10 mM	6	168 ± 12	245 ± 20	132 ± 8	9.0 ± 0.7	28 ± 1.7	95 ± 6	+95 ± 6	100

the heart was not affected by the presence of either acetate or pyruvate. The most striking effects of acetate or pyruvate on glucose metabolism were on $^{14}CO_2$ production and glycogen synthesis. The production of $^{14}CO_2$ was inhibited by 85%, while the incorporation of glucose counts into glycogen was stimulated 6-fold in conjunction with a net increase of cardiac glycogen.

The addition of insulin alone to hearts perfused with 5 mM glucose produced a large increase of glucose uptake, lactate formation, $^{14}CO_2$ production, and glycogen synthesis (Table I). Incorporation of glucose counts into CO_2 and glycogen accounted for 42 and 20%, respectively, of the medium count decrease. Most of the remaining counts removed from the medium were recovered as ^{14}C intermediates in the heart. The formation of these compounds from glucose was stimulated 3- to 4-fold by insulin. Acetate or pyruvate in the presence of insulin decreased glucose uptake to about one-half the values with insulin alone, and lactate formation in the presence of acetate was decreased in proportion to the glucose uptake. The medium count decrease was diminished 42% by both substrates. A comparison of the values for glucose uptake in the presence of pyruvate and insulin with those for the medium count decrease show a difference of 36 μmoles of glucose equivalents per g (dry weight) per hour (Table I). This value gives an estimate of the lactate formation from glucose in the presence of pyruvate, and represents about 30% of the total lactate formation. As in the absence of insulin, acetate or pyruvate in the presence of insulin almost completely inhibited glucose oxidation to $^{14}CO_2$. Glycogen synthesis, on the other hand, was increased approximately 2-fold, and 67 to 72% of the counts removed from the medium were converted to glycogen. Net glycogen synthesis closely paralleled the incorporation of glucose counts into glycogen. The incorporation of counts into ^{14}C intermediates in the heart was decreased 42% by acetate and 51% by pyruvate. The total recovery of isotope in all experiments was approximately quantitative.

Lipid Content of Hearts—The total lipid content of the hearts used in this investigation was of the order of 23 mg per heart. Most of the lipid was probably unavailable as respiratory fuel, but acetate had a lipid-sparing action, since six hearts at the end of 60 minutes of perfusion with medium containing 5 mM glucose and 10 mM acetate had 197 ± 4.0 mg per g (dry weight) of total lipid compared with 179 ± 3.2 mg per g (dry weight) when acetate was omitted from the medium. Taking the difference between these values, acetate spared the utilization of 18 ± 5.6 mg per g (dry weight) of heart lipid. The oxidation of this quantity of lipid requires about 1600 μmoles per g (dry weight) of oxygen, which is sufficient to account for 90% of the total oxygen consumption of the perfused rat heart (13). This is consistent with the observation that the $^{14}CO_2$ formation from glucose, with glucose as the sole exogenous substrate, accounts for only 14% of the oxygen consumption. Acetate also increased the incorporation of ^{14}C-glucose into lipids, from 0.29 ± 0.07 to 0.71 ± 0.05 μmole of glucose equivalent per g (dry weight) during the 60-minute perfusion period. Saponification of the lipid yielded very few counts in the fatty acids, indicating that most of the counts in the lipid were probably derived from ^{14}C-α-glycerophosphate. Hearts perfused with medium containing 5 mM glucose released a small amount of glycerol into the perfusion medium, but the amount released was not affected by the presence of acetate or pyruvate, being 4.6 ± 0.8 μmoles per g (dry weight) per hour for the control hearts, 4.6 ± 0.2 μmoles per g (dry weight) per hour in the presence of acetate, and 4.4 ± 0.6 μmoles per g (dry weight) per hour in the presence of pyruvate (six hearts in each group).

Intracellular Glucose and Adenine Nucleotide Concentrations—With control hearts in the absence of insulin, the glucose space was found to be slightly less than the sorbitol space, showing that intracellular glucose levels were too low to be detected. Neither acetate nor pyruvate in the absence of insulin affected the volume distribution of glucose (Table II). In accordance with previous observations (20, 32), insulin increased the glucose space to values greater than the extracellular space. If a uniform distribution of glucose throughout the intracellular space was assumed, the intracellular glucose concentration was approximately 30% of the perfusate glucose concentration and may have been even higher, since it has been reported that only 75% of the intracellular water is available for sugar distribution (20). Acetate or pyruvate in the presence of insulin caused a 2-fold increase of intracellular glucose, and concentrations at least 50% of those in the perfusate were attained. Insulin decreased the volume of intracellular water by 10% in these experiments ($p < 0.01$).

Acetate or pyruvate, either in the presence or absence of in-

TABLE II

Effects of acetate and pyruvate on glucose space in perfused rat heart

Hearts were perfused for 15 minutes with 5 mM glucose, transferred to recirculation circuits for a further 30 minutes of perfusion, and then rapidly frozen. Values shown are means ± standard error of the mean.

Additions to 5 mM glucose	No. of hearts	Intracellular water	Glucose space / Sorbitol space	Intracellular glucose	
		μl/g (dry wt)	%	mM	μmoles/g (dry wt)
No insulin					
Control	8	2071 ± 24	92 ± 1.3		
Acetate, 10 mM	6	2181 ± 57	93 ± 1.3		
Pyruvate, 10 mM	10	2118 ± 35	90 ± 0.7		
Insulin, 2 milliunits per ml					
Control	8	1884 ± 32	113 ± 1.3	1.05 ± 0.11	1.98 ± 0.20
Acetate, 10 mM	6	1880 ± 126	124 ± 1.5	1.91 ± 0.26	3.86 ± 0.28
Pyruvate, 10 mM	10	1962 ± 35	125 ± 2.2	2.20 ± 0.15	4.34 ± 0.35

TABLE III

Effects of acetate and pyruvate on levels of adenine nucleotides in perfused rat heart

The perfusion conditions were similar to those of Table II.

Additions to 5 or 10 mM glucose	ATP*	ADP†	AMP†
		μmoles/g (dry wt)	
No insulin			
Control	21.7 ± 0.3 (8)‡	2.49 ± 0.11 (10)	0.35 ± 0.07 (10)
Acetate, 10 mM	22.4 ± 0.4 (6)	2.05 ± 0.29 (5)	0.70 ± 0.17 (5)
Pyruvate, 10 mM	22.9 ± 0.5 (10)	2.45 ± 0.09 (6)	0.32 ± 0.03 (6)
Insulin, 2 milliunits per ml			
Control	22.4 ± 0.4 (8)	1.82 ± 0.27 (4)	0.15 ± 0.04 (4)
Acetate, 10 mM	21.6 ± 1.0 (6)	2.44 (2)	0.48 (2)
Pyruvate, 10 mM	22.8 ± 0.4 (10)		

* Perfusion for 30 minutes.

† Perfusion for 15 minutes.

‡ Mean ± standard error with number of hearts in parentheses.

TABLE IV

Effects of acetate and pyruvate on levels of glycolytic intermediates in perfused rat heart

The perfusion conditions were as described in Table II. Values shown are means ± standard error of the mean.

Additions to 5 mM glucose	No. of hearts	Glucose-6-P	Fructose-6-P	Fructose-1,6-di-P	Triose-P	Pyruvate
		mμmoles/g (dry wt)				
No insulin						
Control	8	270 ± 16	63 ± 7	47 ± 5	79 ± 10	130 ± 8
Acetate, 10 mM	6	990 ± 51	165 ± 12	36 ± 3	65 ± 5	140 ± 9
Pyruvate, 10 mM	8	760 ± 60	120 ± 12	160 ± 22	482 ± 58	
Insulin, 2 milliunits per ml						
Control	8	1930 ± 56	361 ± 18	85 ± 15	133 ± 9	300 ± 31
Acetate, 10 mM	6	2620 ± 180	456 ± 46	68 ± 14	125 ± 20	160 ± 15
Pyruvate, 10 mM	8	2550 ± 82	478 ± 35	181 ± 36	482 ± 70	

sulin, had no significant effect on the tissue levels of ATP or ADP. Acetate, but not pyruvate, increased the levels of AMP 3-fold (Table III). Although pyruvate had little effect on the tissue levels of the adenine nucleotides, the creatine content decreased from 27.1 ± 1.0 to 19.5 ± 0.9 μmoles per g (dry weight) (4 hearts, $p = 0.001$) upon the addition of 10 mM pyruvate to hearts perfused with 10 mM glucose, while creatine phosphate increased from 31.4 ± 1.8 to 45.1 ± 1.4 μmoles per g (dry weight) ($p = 0.001$).

Tissue Levels of Glycolytic Intermediates—The stationary state levels of glucose-6-P, fructose-6-P, fructose-1,6-di-P, triose-P, and pyruvate in hearts after 30 minutes of perfusion in the presence and absence of acetate or pyruvate are shown in Table IV. These values were determined by the spectrophotometric assay procedure with 1.0 ml. of extract. In the absence of insulin, acetate increased the tissue levels of glucose-6-P and fructose-6-P 3- to 4-fold, decreased the level of fructose-1,6-di-P slightly, but had no significant effect on the levels of triose-P or pyruvate. The addition of pyruvate, on the other hand, increased glucose-6-P and fructose-6-P levels 2- to 3-fold, and also greatly increased the levels of fructose-1,6-di-P and triose-P. Insulin alone increased glucose-6-P and fructose-6-P levels 6- to 7-fold, while

the levels of fructose-1,6-di-P, triose-P, and pyruvate were ...ased approximately 2-fold (Table IV). The addition of ...r acetate or pyruvate in the presence of insulin produced a further 32 to 36% increase of the hexose monophosphates ($p < 0.001$). However, as in the absence of insulin, while acetate had little effect on the levels of fructose-1,6-di-P and triose-P, these intermediates were greatly elevated by the addition of pyruvate.

The results of these experiments indicated that, in the presence of acetate, glycolysis was inhibited by decreased activity of phosphofructokinase, while pyruvate also appeared to act at a site further down the glycolytic pathway. In order to confirm the effects obtained with acetate, and to determine the site or sites of action of pyruvate, the entire pattern of glycolytic intermediates was measured in a series of hearts by fluorometric assay procedures. These results are presented graphically in Fig. 2. The glycolytic intermediates are plotted in sequence against the percentage change of the component with respect to the mean control value. Control hearts were perfused for 15 minutes with 10 mM glucose in the absence of insulin. Other hearts were analyzed 15 minutes after the addition of 10 mM acetate, or 5 minutes after the addition of 10 mM pyruvate to a similar perfusate. These results clearly show an inhibition of phosphofructokinase after the addition of acetate, as shown by an increase in the levels of hexose monophosphates, and a decrease in the levels of fructose-1,6-di-P and the other intermediates in the linear sequence of the enzymic reactions of the glycolytic pathway. The levels of α-glycero-P showed no change upon addition of acetate, but since the dihydroxyacetone-P levels decreased, the ratio of α-glycero-P to dihydroxyacetone-P increased by 50%. The large increase of fructose-1,6-di-P and particularly the triose-P after the addition of pyruvate, compared with the much smaller increases in 3-P-glyceric acid and 2-P-glyceric acid, in addition to the increase of hexose monophosphates, indicate inhibition at an enzymic site between glyceraldehyde-3-P and 3-P-glyceric acid as well as at phosphofructokinase. The kinetics of the changes in the levels of fructose-1,6-di-P, triose-P, and 3-P-glyceric acid after pyruvate addition are shown in Fig. 3. It may be seen that 3-P-glycerate increased by 45% within 30 seconds, and remained constant on further perfusion, while fructose-1,6-di-P and triose-P did not begin to increase until after 60 seconds, and reached maximum values 5 minutes after pyruvate addition.

The ratio of α-glycero-P to dihydroxyacetone-P decreased rapidly after the addition of pyruvate to hearts perfused with glucose and insulin (Fig. 4), and a constant value was attained within 2 minutes. Similar changes were observed in the absence of insulin. The time for half-maximal change was of the order of 30 seconds. The ratios of the concentrations of the three NAD-linked substrate couples, lactate-pyruvate, α-glycero-P-dihydroxyacetone-P, and malate-oxaloacetate in the heart or perfusate 15 minutes after the addition of either acetate or pyruvate to hearts perfused with glucose are shown in the first three columns of Table V. The ratio of each of the substrate couples increased in the presence of acetate and decreased in the presence of pyruvate, compared with control hearts perfused with glucose alone.

The ratio of these metabolite pairs has frequently been used

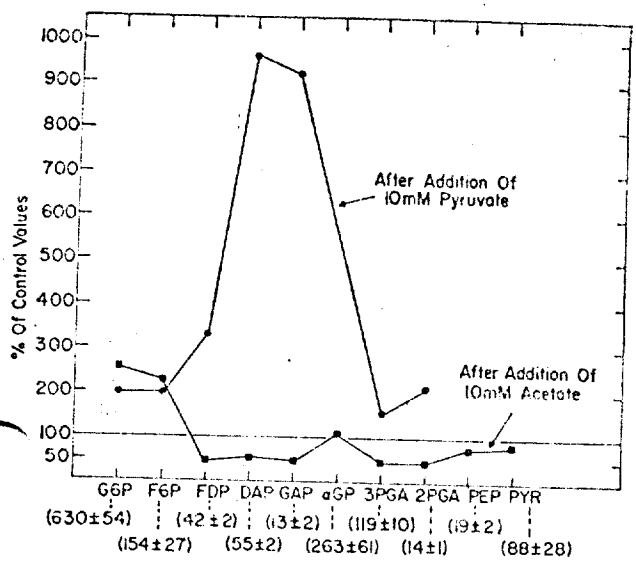

FIG. 2. Effects of acetate and pyruvate (*PYR*) on the levels of glycolytic intermediates in the perfused rat heart. Values of the glycolytic intermediates 15 minutes after the addition of 10 mM acetate and 5 minutes after the addition of 10 mM pyruvate are expressed as a percentage of control values obtained in hearts perfused for 15 minutes with glucose alone. Control values are shown in brackets, and are expressed in millimicromoles per g (dry weight) ± standard error of the mean, 4 to 10 hearts per group. Changes in the levels of all the intermediates except pyruvate and α-glycerophosphate (*α-GP*) were statistically significant ($p < 0.01$). Other abbreviations: *G6P*, glucose-6-P; *F6P*, fructose-6-P; *DAP*, dihydroxyacetone-P; *3PGA*, 3-P-glyceric acid; *2+PGA*, 2-P-glyceric acid; *PEP*, phosphoenolpyruvate.

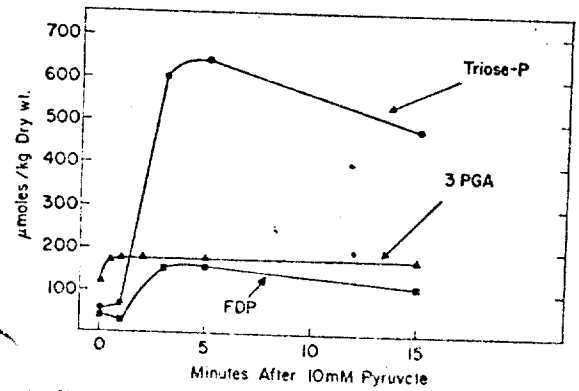

FIG. 3. Concentrations of triose phosphates, 3-P-glyceric acid (*3PGA*), and fructose-1,6-di-P (*FDP*) after the addition of 10 mM pyruvate to rat hearts previously perfused for 15 minutes with 10 mM glucose.

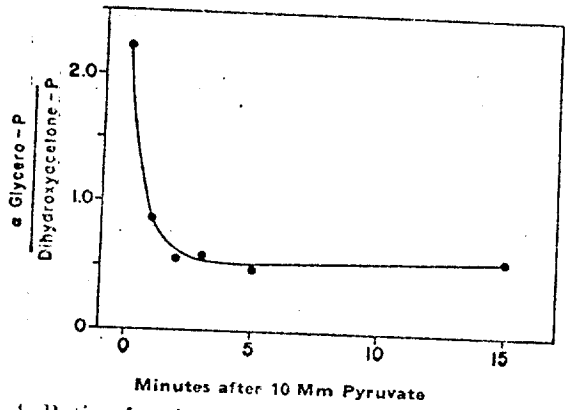

FIG. 4. Ratio of α-glycero-P to dihydroxyacetone-P after the addition of 10 mM pyruvate to rat hearts perfused with 5 mM glucose and 2 milliunits of insulin per ml.

to estimate the ratio of the free NAD to NADH in the extramitochondrial compartment of intact organs and cells by a method first introduced in experiments on yeast by Holzer, Schulz, and Lynen (33), and applied in some detail to rat liver by Bücher *et al.* (34–36). The validity of the method rests on the following assumptions: (*a*) the reactants of the three enzymes lactic dehydrogenase, α-glycero-P dehydrogenase, and malic dehydrogenase are each in thermodynamic equilibrium in the intact cell; (*b*) equilibrium is established with a common pool of pyridine nucleotides; (*c*) the concentrations of metabolites in the extramitochondrial compartment are represented by levels determined in a whole tissue extract; (*d*) the intracellular pH is 7.0 and does not change with the experimental conditions (37). If these assumptions involve no gross errors, the ratio of the metabolite couples for a given pair of reactions should equal the ratio of the mass action equilibrium constants of the corresponding NAD-linked reactions (34, 35). Table V shows that these relationships hold reasonably well for the lactic dehydrogenase and α-glycero-P dehydrogenase reactions in control hearts, and hearts perfused with glucose and acetate, but not for hearts perfused with glucose and pyruvate. The malic dehydrogenase reaction compared with either the lactic dehydrogenase or the α-glycero-P dehydrogenase reactions showed considerably greater deviations from theoretical behavior.

TABLE V

Ratio of substrate oxidation-reduction couples lactate-pyruvate, α-glycerophosphate-dihydroxyacetone phosphate and malate-oxaloacetate in perfused rat heart after addition of acetate or pyruvate

All hearts were perfused for 15 minutes. The values for the concentration of the intermediates were taken from Fig. 2, Table VIII, and from data not presented elsewhere. The ratios of the equilibrium constants for the NAD-linked metabolite pairs are (35):

$$\frac{\text{Lact}^*}{\text{Pyr}} \times \frac{\text{DAP}}{\alpha\text{-GP}} = 1.7, \quad \frac{\text{Mal}}{\text{OAA}} \times \frac{\text{DAP}}{\alpha\text{-GP}} = 9.1, \quad \frac{\text{Mal}}{\text{OAA}} \times \frac{\text{Pyr}}{\text{Lact}} \times 5.4$$

Additions to 10 mM glucose	$\frac{\text{Lact}}{\text{Pyr}}$†	$\frac{\alpha\text{-GP}}{\text{DAP}}$	$\frac{\text{Mal}}{\text{OAA}}$	$\frac{\text{Lact}}{\text{Pyr}} \times \frac{\text{DAP}}{\alpha\text{-GP}}$	$\frac{\text{Mal}}{\text{OAA}} \times \frac{\text{DAP}}{\alpha\text{-GP}}$	$\frac{\text{Mal}}{\text{OAA}} \times \frac{\text{Pyr}}{\text{Lact}}$
Control	5.3	4.9	90	1.1	18	17
Acetate, 10 mM	17.7	7.3	556	2.4	77	31
Pyruvate, 10 mM	0.1	0.76	39	0.13	51	390

* The abbreviations used in the tables are: Lact, lactate; Pyr, pyruvate; α-GP, α-glycero-P; DAP, dihydroxyacetone-P; Mal, malate; OAA, oxaloacetate.

† Perfusate concentration ratio.

TABLE VI

Effects of acetate and pyruvate on extramitochondrial NAD-NADH potential in perfused rat heart

The observed ratios of lactate-pyruvate, α-glycero-P-dihydroxyacetone-P, and malate-oxaloacetate given in Table V were used to calculate the potential of extramitochondrial NAD-NADH by means of the formula

$$E_h = E_0' + \frac{RT}{2F} \ln \frac{[\text{Ox}]}{[\text{Red}]}$$

The equilibrium constants (pH 7.0, 37°) for lactic dehydrogenase, α-glycerophosphate dehydrogenase, and malic dehydrogenase were taken to be 19,000, 11,000, and 102,000, respectively (35), and −335 mv was used for the midpotential (E_0') of the NAD-NADH system at 37° (38).

Additions to 10 mM glucose	Potential of extramitochondrial NAD-NADH calculated from ratios		
	$\frac{\text{Lact}}{\text{Pyr}}$	$\frac{\alpha\text{-GP}}{\text{DAP}}$	$\frac{\text{Mal}}{\text{OAA}}$
	mv		
Control	−228	−234	−243
Acetate, 10 mM	−243	−239	−266
Pyruvate, 10 mM	−176	−209	−232

The extramitochondrial NAD:NADH potentials, calculated from the observed ratios of lactate to pyruvate, α-glycero-P to dihydroxyacetone-P, and malate to oxaloacetate, are shown in Table VI. It may be seen that values calculated from the three NAD-dependent systems for control hearts are in reasonably close agreement with each other and provide a mean of −235 mv, which is similar to the average potential calculated for a number of tissues by Klingenberg and Bücher (38). Despite the discrepancies between the potentials calculated in the presence of acetate and pyruvate, it is clear that acetate changes the cytoplasmic potential towards a more reduced state, while pyruvate produces a more oxidized state. The lactic dehydrogenase and α-glycero-P dehydrogenase reactions are probably close to equilibrium under conditions of normal glycolytic flux, and decreased flux produced by the addition of acetate, but lactic dehydrogenase would not appear to be sufficiently active to maintain equilibrium

TABLE VII

Effects of acetate and pyruvate on levels of pyridine nucleotides in perfused rat heart

The perfusion conditions were as described in Fig. 2. Values shown are means ± standard error of the mean.

Additions to 5 mM glucose	No. of hearts	NAD	NADH	NADP	NADPH
		mμmoles/g (dry wt)			
Perfusion for 15 min					
Control	4	3980 ± 64	236 ± 13	196 ± 21	295 ± 12
Acetate, 10 mM	4	4120 ± 135	258 ± 7	181 ± 10	290 ± 6
Perfusion for 5 min					
Control	6	3970 ± 47	177 ± 42	266 ± 16	183 ± 30
Pyruvate, 10 mM	6		760 ± 51	193 ± 5	277 ± 9

the presence of excess pyruvate. It should be pointed out, however, that lactic dehydrogenase may in fact be closer to equilibrium than suggested from the data in Tables V and VI, since the figures given refer to the concentration of lactate and pyruvate in the perfusate, rather than in the tissue, where the ratio of lactate to pyruvate may be higher due to diffusion gradients. The lack of correspondence between the potentials calculated from the α-glycero-P dehydrogenase and the malate dehydrogenase reactions indicates that values of malate and oxaloacetate obtained from the analysis of whole tissue extracts are of limited use in calculating the cytoplasmic oxidation-reduction potential.

Tissue Levels of Pyridine Nucleotides—The total amounts of oxidized and reduced pyridine nucleotides in hearts after the addition of acetate or pyruvate are shown in Table VII. Acetate produced no significant changes in the levels of these cofactors, but pyruvate increased NADH levels by 330%, and NADPH levels by 51% with a corresponding decrease of NADP. In control hearts, the total amount of oxidized and reduced diphosphopyridine nucleotides exceeded that of the triphosphopyridine nucleotides by a factor of 9. The ratio of oxidized to reduced diphosphopyridine nucleotide in control hearts was about 20, while that of triphosphopyridine nucleotide was about unity.

The kinetics of the changes of the oxidized pyridine nucleotides after addition of pyruvate are shown in Fig. 5. The levels of NAD and NADP started to decrease shortly after pyruvate addition, with the rate of decrease being most rapid between the 1st and 2nd minute. This was followed by a progressive, small increase of NADP between the 3rd and 10th minute of perfusion with pyruvate, while NAD levels remained low.

The kinetics of the changes of the reduced pyridine nucleotides after addition of pyruvate were determined from the fluorescence emission of the intact beating heart. Fig. 6 (*upper trace*) shows a recording of the time course of fluorescence intensity changes as observed with the microfluorometer. The *lower tracing* in Fig. 6 records the tissue oxygen tension. The heart at the beginning of the experiment was perfused with 20 ml of perfusate containing 10 mM glucose. Deoxygenation of the perfusate produced an increase of fluorescence, as shown by a downward deflection of the upper trace, and a decrease of the tissue oxygen tension from 130 mm Hg to a value slightly greater than zero. Upon reoxygenation of the perfusate, the fluorescence intensity and the tissue p_{O_2} returned to their original levels. Addition of 100 μmoles of pyruvate to the upper reservoir of the perfusion apparatus produced a linear increase of fluorescence after a short delay due to the time lag necessary for the pyruvate to reach the heart, and the maximum effect was obtained 90 seconds after the start of the fluorescence increase. This was two-thirds as great as the change with anoxia. Analysis of hearts perfused under similar conditions for reduced pyridine nucleotides show that the fluorescence increase was primarily due to the formation of NADH (*cf.* Table VII).

Arsenite (0.1 mM final concentration), an inhibitor of dihydrolipoyl dehydrogenase (39), completely reversed the increase

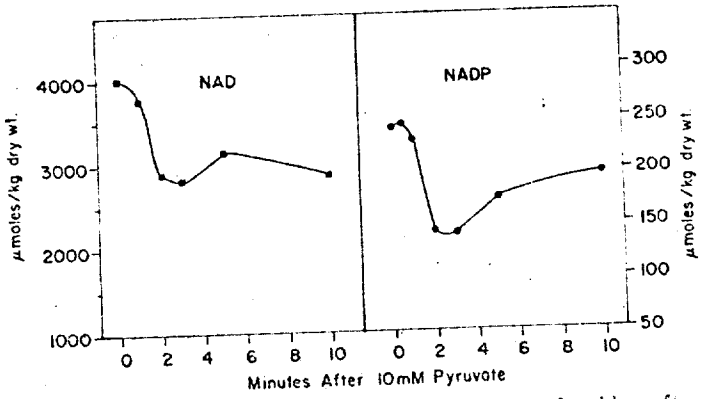

FIG. 5. Concentrations of oxidized pyridine nucleotides after the addition of 10 mM pyruvate to rat hearts perfused with 10 mM glucose.

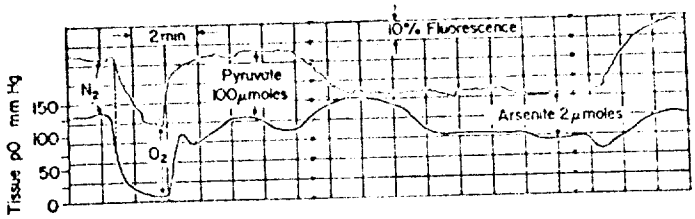

FIG. 6. Comparison of the fluorescence intensity of the intact beating heart (*upper trace*) with the tissue oxygen tension (*lower trace*). An increase of fluorescence is recorded as downward deflection of the trace. Glucose, 10 mM, was added to the perfusate (20 ml) 15 minutes before the addition of pyruvate.

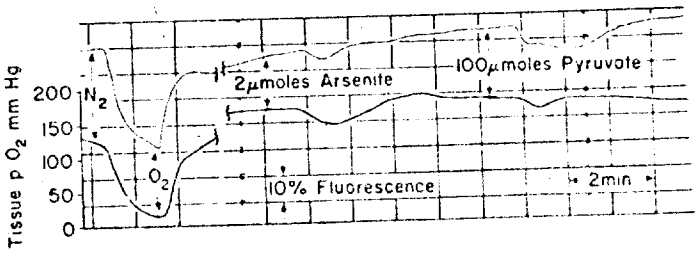

FIG. 7. Effect of pyruvate after prior addition of arsenite on the fluorescence intensity (*upper trace*) and the tissue oxygen tension (*lower trace*) of hearts perfused with 20 ml of fluid. An increase of fluorescence is recorded as a downward deflection of the trace.

TABLE VIII

Effects of acetate and pyruvate on levels of citric acid cycle intermediates in perfused rat heart

Hearts were perfused with 15 ml of fluid for 15 minutes in recirculation circuits and rapidly frozen. Values shown are means ± standard error of the mean of four or more hearts.

Additions to 10 mM glucose	Citrate	Isocitrate	α-Ketoglutarate	Malate	Oxaloacetate
			mμmoles/g (dry wt)		
Control	347 ± 18	78 ± 3	161 ± 12	451 ± 20	5.0 ± 0.7
Acetate, 10 mM	3500 ± 180	276 ± 4	340 ± 25	667 ± 69	1.2 ± 0.2
Pyruvate, 10 mM	7870 ± 450	414 ± 49	2720 ± 220	6250 ± 510	159 ± 12

TABLE IX

Effects of acetate and pyruvate on amino acid levels in perfused rat heart

Conditions of perfusion were the same as in Table VIII.

Additions to 10 mM glucose	Glutamate	Aspartate	Glutamine	Asparagine
	μmoles/g (dry wt)			
Control	29.3 ± 1.5	9.22 ± 0.32	32.5 ± 1.4	3.02 ± 0.31
Acetate, 10 mM	35.4 ± 0.8	3.41 ± 0.18	32.3 ± 1.2	3.04 ± 0.17
Pyruvate, 10 mM	15.4 ± 0.5	9.71 ± 0.57	26.4 ± 2.7	3.63 ± 0.72

of fluorescence produced by pyruvate, and the final fluorescence intensity was lower than the initial steady state level. The tissue p_{O_2} increased transiently after pyruvate addition and returned to an equilibrium level slightly lower than the initial, possibly owing to a small increase of oxygen consumption by the heart. The addition of arsenite caused only a small increase of tissue p_{O_2}, indicating that the total oxygen consumption of the heart was not greatly inhibited. Fig. 7 shows that if arsenite was added to the perfusate prior to pyruvate, the normal increase of fluorescence produced by pyruvate was much smaller and lasted for only 2 minutes.

Tissue Levels of Citric Acid Cycle Intermediates, Glutamate, and Aspartate—The addition of either acetate or pyruvate to hearts perfused with medium containing glucose produced large changes in the total content of intermediates of the citric acid cycle (Table VIII), and the amino acids glutamate and aspartate (Table IX). The effects of adding acetate were, however, different from the effects following the addition of pyruvate. Acetate increased citrate 10-fold, α-ketoglutarate 2-fold, malate by 50%, and glutamate by 17%, while oxaloacetate was decreased by 75% and aspartate by 63%. Pyruvate, on the other hand, increased citrate 22-fold, α-ketoglutarate 17-fold, malate 14-fold, and oxaloacetate 32-fold, while glutamate was decreased by 48%, and there was no effect on aspartate. Isocitrate levels were 22, 8, and 5%, respectively, of the citrate levels in control and in acetate- and pyruvate-treated hearts. Glutamate and aspartate remained approximately in equilibrium with their respective keto acids, despite large changes in the total amounts of these intermediates found in the heart. The ratios of the products to the reactants of the glutamic-oxaloacetate transaminase reaction were 10, 27, and 11 for control, acetate-treated, and pyruvate-treated hearts, respectively, compared with an equilibrium constant of 8 at 37.5° (40). The levels of glutamine and asparagine showed only small changes upon the addition of pyruvate, and no change upon the addition of acetate (Table IX).

DISCUSSION

The present experiments with ^{14}C-glucose have confirmed previous observations (10) that pyruvate and acetate decreased the glucose utilization of the perfused rat heart in the presence of insulin. Inhibition of glucose uptake was less pronounced in the absence than in the presence of insulin, when flux through the glycolytic pathway was considerably increased, but in either case the oxidation of glucose to CO_2 was decreased to a greater extent than glucose uptake, and the principal fate of the glucose removed from the medium was conversion to lactate and glycogen. In the absence of insulin, free glucose was not detected in the intracellular fluid, and the addition of acetate or pyruvate did not increase the glucose space. These findings indicate that glucose uptake was limited by the rate of transport across the cell membrane. Pyruvate, but not acetate, acetoacetate (1), or lactate (41), decreased the rate of glucose uptake in the absence of insulin; and a similar effect of octonoate, but not of palmitate, has been reported by Bowman (42), suggesting that some substrates may interfere directly with the transport process.

Addition of either acetate or pyruvate to the perfusate in the presence of insulin resulted in a decreased rate of glucose phosphorylation, as shown by a decreased rate of glucose uptake, and an increase of free intracellular glucose. Net transport of glucose into the cell was decreased as a result of the elevated intracellular levels of glucose. Similar effects have been reported with long chain fatty acids and ketone bodies (2, 42). A decreased rate of glucose phosphorylation has also been observed in perfused hearts from alloxan-diabetic rats, and hypophysectomized-diabetic rats treated with growth hormone and cortisone (43). The decreased hexokinase activity was shown to be associated with elevated tissue levels of glucose-6-P. In the present study, the decreased rate of glucose phosphorylation was likewise associated with increased levels of glucose-6-P. Since glucose-6-P is a potent inhibitor of hexokinase in the cell-free system (44), it has frequently been proposed that a similar type of inhibition may control the rate of glucose phosphorylation in the intact cell. However, glucose phosphorylation was not inhibited by acetate or pyruvate in the absence of insulin to a sufficient degree to cause the accumulation of free glucose, despite a 3-fold increase of glucose-6-P levels. Furthermore, insulin alone increased the mean intracellular concentration of glucose-6-P to about 1 mM, a level theoretically sufficient to inhibit heart muscle hexokinase by 90%, but no inhibition of glucose phosphorylation was observed. An increased rate of hexokinase activity associated with substantial increase of glucose-6-P has been observed in frog skeletal muscle during anoxia (45) and with perfused rat heart during epinephrine stimulation (46). Clearly, factors other than the tissue level of glucose-6-P regulate hexokinase activity in the intact cell. Rose, Warms, and O'Connell (47) have shown that inorganic phosphate was able to relieve partially the glucose-6-P inhibition of hexokinase from human erythrocytes. Whether this type of mechanism is important in animal tissues has not yet been established experimentally.

The increased glucose-6-P levels found after the addition of acetate or pyruvate, both in the presence and absence of insulin, were associated with an increased incorporation of ^{14}C-glucose into glycogen, in conjunction with a rise of total cardiac glycogen. Glucose-6-P presumably promoted glycogen synthesis by activation of uridine diphosphate glucose-α-glucan transferase (48, 49). Inhibition of glycolysis thus caused a diversion of most of the glucose which entered the cell from oxidation to glycogen synthesis. This effect was particularly striking in the absence of insulin.

The accumulation of fructose-1,6-di-P and triose-P in the presence of pyruvate, and the depletion of these intermediates in the presence of acetate, relative to the controls, suggests at first sight that different mechanisms control the glycolytic flux with the two substrates. Interpretation of the results is aided by a consideration of the displacement from equilibrium of the glycolytic reactions. The operation of each step in the intact glycolytic chain may be evaluated by expressing the data in terms of the mass action ratios of the metabolites for each of the

TABLE X

Comparison of mass action ratios with apparent equilibrium constants for glycolytic reactions

Values for the mass action ratios are calculated from the data presented in Tables II, IV, and VI, Fig. 2, and data not presented elsewhere. The direction of each of the reactions is taken in the forward direction of glycolytic flux. The apparent equilibrium constants are taken from values reported in the literature (29, 50–55), and are expressed in liter mole^{-1}.

Reaction	Apparent equilibrium constant	Mass action ratios in the presence of the following substrates		
		Glucose	Glucose + acetate	Glucose + pyruvate
Hexokinase*	$1\text{–}5 \times 10^{3}$	0.08	0.08	0.06
Phosphoglucomutase	5.5×10^{-2}	4×10^{-3}		8×10^{-2}
Phosphoglucose isomerase	0.28	0.24	0.22	0.24
Phosphofructokinase	1.2×10^{3}	0.03	0.005	0.05
Aldolase	$7\text{–}13 \times 10^{-5}$	9×10^{-6}	5×10^{-6}	23×10^{-5}
Triosephosphate isomerase	4×10^{-2}	0.24	0.21	0.23
ALD × TPI†	4×10^{-6}	2.2×10^{-6}	1×10^{-6}	53×10^{-6}
GAPDH × PGK‡	1.5×10^{3}	9	16	0.3
Phosphoglycerate mutase	0.1–0.17	0.12	0.12	0.16
Enolase	4.6–6.3	1.4	2.3	2.5
Pyruvic kinase	$2\text{–}15 \times 10^{3}$	40	56	
Adenylate kinase	0.44	0.82	0.27	0.89

* Values were calculated from experiments conducted in the presence of 2 milliunits of insulin per ml.

† A product of the mass action ratios of aldolase and P-isomerase steps. See text.

‡ The α-glycerophosphate dehydrogenase reaction was used to obtain the NAD-NADH ratio, and a phosphate concentration of 4 mM was assumed (43). The abbreviation is similar to that described in the footnote above.

individual steps in the glycolytic sequence, and comparing these ratios with the known equilibrium constants (55). By convention, the reaction products are made the numerators of the ratios. These data are shown in Table X. It may be seen that hexokinase, phosphofructokinase, and pyruvic kinase are far displaced from equilibrium in the intact cell, whereas equilibrium is approximated at the following steps: phosphoglucomutase, phosphoglucose isomerase, phosphoglycerate mutase, enolase, and adenylate kinase. Triosephosphate isomerase and aldolase are maintained somewhat far from equilibrium, the ratio of dihydroxyacetone-P to glyceraldehyde-3-P, for instance, being approximately 4:1, instead of 24:1 if equilibrium conditions were established. A similar lack of equilibrium at the triose-P isomerase step has been observed in Ehrlich ascites tumor cells (55). A computer simulation of aerobic glycolysis in this tissue (56) showed that the flux pattern was incompatible with the observed ratio of dihydroxyacetone-P and glyceraldehyde-3-P unless some form of compartmentation was invoked, and it was suggested that aldolase and triose-P isomerase may be in close association within the cell. The observed data were explained on the assumption that part of the dihydroxyacetone-P remained bound to aldolase for some time after cleavage of the fructose-1,6-di-P in such a position that it interfered with the action of the isomerase. This type of explanation may have general validity since Table X shows that the product of the mass action ratios of the aldolase and triose-P isomerase steps (ALD × TPI, Table X) are relatively close to the equilibrium value.

No experimental values for 1,3-di-P-glycerate are available, so that it is not possible to calculate the mass action ratios of glyceraldehyde-P dehydrogenase and P-glyceric kinase individually, but as shown in Table X, the product of the mass action ratios of these reactions is far displaced from equilibrium. An indirect assessment of the deviation from equilibrium of each of these reactions can be made by calculating the expected level of 1,3-di-P-glycerate on the assumption that either one of the enzyme partners is close to equilibrium. By assuming equilibrium at the P-glycerate kinase steps, and from the observed values of 3-P-glycerate, ADP, and ATP, and an equilibrium constant of 3×10^{3} at pH 7 (50), equilibrium values for the concentration of 1,3-di-P-glycerate of 0.7 μM are obtained for the control hearts. Corresponding equilibrium values calculated from the glyceraldehyde-P dehydrogenase reaction are 30 μM, with a value of 2250 for the ratio of NAD to NADH (calculated from the α-glycerophosphate dehydrogenase reaction), a phosphate concentration of 4 mM (43), and an equilibrium constant of 0.5 liter mole^{-1} (53). If the higher concentration of 1,3-di-P-glycerate was present in the heart, it should have been readily detectable with the sensitive assay procedure used, but attempts to measure this intermediate were unsuccessful, despite rapid preparation and assay of the extracts. The lower figure, on the other hand, is of the same order as the values reported by Hohorst, Reim, and Bartels (57) in rat abdominal muscle. Disequilibrium at the dehydrogenase step thus appears more likely than at the phosphoglyceric kinase step. It should perhaps be pointed out, however, that these calculations are based on a lack of compartmentation of cytoplasmic DPNH, or in other words, that glyceraldehyde-P dehydrogenase and α-glycero-P dehydrogenase react with the same pool of pyridine nucleotides. At the present time, this seems to be a reasonable assumption.

These conclusions are in general agreement with studies of glycolysis in brain (58), ascites tumor cells (55), and stimulated rat abdominal muscle (see discussion of Bücher and Rüssmann (59)), except for some uncertainty regarding the glyceraldehyde-P dehydrogenase and P-glyceric kinase steps. There is a considerable loss of free energy at those enzymic sites which are maintained far from equilibrium during glycolytic flux. Generally, these reactions are not appreciably reversible and act as potential control points. The free energy change for reactions close to equilibrium is small, on the other hand, and these steps being readily reversible are not normally susceptible to control.

On the basis of the crossover theorem of Chance *et al.* (60), it is clear from the data of the intermediate levels presented in Fig.

that phosphofructokinase exerts absolute control over the glycolytic flux in hearts perfused in the presence of acetate. Fatty acids and ketone bodies seem to behave similarly (2). Decreased activity of phosphofructokinase has also been demonstrated in hearts from alloxan-diabetic rats perfused with glucose and insulin (43). Acute alloxan diabetes is characterized by elevated plasma levels of free fatty acids and ketone bodies, and, as with starvation (10, 41), defective carbohydrate utilization is manifested in hearts when removed from the animal and transferred to a glucose medium. Endogenous fat is then the major fuel, and inhibition at the phosphofructokinase site appears to be associated with enhanced respiration of fatty acids.

After the addition of pyruvate to hearts perfused with glucose, either in the presence (61) or the absence of insulin, no crossover point can be identified from the changes in the levels of the glycolytic intermediates. These experiments illustrate that interaction at several control sites determines the steady state levels of the glycolytic intermediates. The hexose phosphates increased immediately after the addition of pyruvate (61) and remained elevated, while fructose-1,6-di-P decreased slightly during the first 1 to 2 minutes and subsequently increased along with the triose phosphates. These changes indicate an initial severe inhibition of phosphofructokinase followed by a partial deinhibition as fructose-1,6-di-P accumulates. The large increase of triose-P (900%) compared with the small increase of 3-P-glyceric acid (56%) indicates decreased activity also of either glyceraldehyde-P dehydrogenase or P-glyceric kinase, as seen from Table X, the product of the mass action ratios of these reactions in the presence of pyruvate is much further displaced from equilibrium than in the controls. There appears to be no lack of phosphate acceptor for the P-glyceric kinase reaction, suggesting that the dehydrogenase may be the enzyme affected. This possibility is supported by the arguments given above, and by the kinetic characteristics of glyceraldehyde-P dehydrogenase, which is strongly inhibited by its product 1,3-di-P-glycerate with a K_i of 0.8 μM (62). Assuming equilibrium at P-glyceric kinase, the addition of pyruvate increased calculated equilibrium values of 1,3-di-P-glycerate from 0.17 to 0.3 μM. These values are sufficiently close to the inhibition constant for the glyceraldehyde-P dehydrogenase reaction that relatively small changes of 1,3-di-P-glycerate would be expected to have a large effect on the reaction velocity. Decreased activity of pyruvic kinase may be responsible for the immediate increase of 3-P-glycerate since this enzyme is normally under considerable inhibition from the high concentration of ATP prevailing in the cell and is also inhibited by high pyruvic concentrations (51, 63). Hence, a further decrease of activity caused by the addition of pyruvate might be expected to increase the steady state concentrations of the intermediates between 1,3-di-P-glycerate and P-enolpyruvate since these enzymic steps remain close to equilibrium.

In summary, phosphofructokinase appears to be the major rate-controlling enzyme of the glycolytic sequence in rat heart, and decreased activity at this step can under some circumstances indirectly control hexokinase activity through product inhibition. The stationary state level of fructose-1,6-di-P in the cell is normally maintained low, but increases in response to a further block downstream. Pyruvic kinase and glyceraldehyde-P dehydrogenase are particularly sensitive inhibitory sites since the reactions catalyzed by these enzymes are maintained far from equilibrium and indirectly control the levels of fructose-1,6-di-P and 1,3-di-P-glycerate. Product inhibition of glyceraldehyde-P dehydrogenase and product activation of phosphofructokinase (64) are thus vital elements participating in the over-all control of glycolysis. In the present experiments with pyruvate, the level of fructose-1,6-di-P does not increase sufficiently relative to the concentrations of the different inhibitors to de-inhibit phosphofructokinase completely, as shown by the maintenance of increased fructose-6-P levels, and the rate of glycolysis remains controlled by phosphofructokinase. In anaerobically glycolyzing *Saccharomyces carlsbergensis*, product activation of phosphofructokinase apparently proceeds further, and the system exhibits a series of damped sinusoidal oscillations of the NADH and glycolytic intermediates (65, 66). Sustained oscillations of the pyridine nucleotide have on occasion been observed with the perfused rat heart (67), indicating that the oscillatory response is probably a universal property of the glycolytic system, but is normally highly damped.

The decreased activity of phosphofructokinase cannot be explained by changes in the phosphate potential (64) since ATP and ADP remained approximately constant, while with acetate (but not pyruvate) AMP (an activator) increased, probably as a result of increased acetate thiokinase activity (68). The only known inhibitor of phosphofructokinase that increased markedly upon the addition of acetate was citrate, in confirmation of similar findings on rat hearts perfused with fatty acids (6, 7). Phosphofructokinase has also been shown to be inhibited in perfused hearts from fluoroacetate-poisoned rats, which have greatly elevated citrate levels (8). The accumulation of citrate presumably accounts also for the phosphofructokinase inhibition after pyruvate addition. The inhibition of brain phosphofructokinase by citrate has been shown by Passonneau and Lowry (69) to be reversed by low concentrations of fructose-1,6-di-P, but apparently the level of fructose-1,6-di-P in hearts perfused with pyruvate did not increase sufficiently to overcome the combined inhibitory effects of ATP and citrate.

Measurements of the NAD concentration in mitochondria and whole cell extracts relative to the cytochrome *c* content have indicated that in heart muscle, NAD is about equally distributed between the cytoplasm and mitochondria (70, 71). The concentration of free NADH in the cytoplasm may thus be calculated from the NAD:NADH ratio derived from the α-glycero-P dehydrogenase reaction, and the NAD concentration in the cytoplasm, on the assumption that 75% of the intracellular water is extramitochondrial. These values are 0.6, 0.9, and 0.08 μM for control, acetate-treated, and pyruvate-treated hearts perfused in the absence of insulin, and are to be compared with values in the order of 100 μM if all the NADH was uniformly distributed throughout the cell water. It may be concluded that in heart muscle under metabolic conditions compatible with those *in vivo*, the bulk of the total NADH is located in the mitochondria. The ratio of the total mitochondrial NAD:NADH for control hearts is thus of the order of 10, which is the same figure as that calculated by Borst (37) for free NAD:NADH from the ratio of acetoacetate to β-hydroxybutyrate (1). In the presence of pyruvate, this ratio approaches unity. Although these calculations are based on several assumptions, and hence may have only qualitative significance, it is clear that the oxidation-reduction state of the mitochondrial NAD:NADH system in the rat heart is very different from that in the cytoplasm, and may change independently. Presumably, the metabolic state of the mitochondria in the perfused rat heart is somewhere be-

tween the fully active State 3 and the resting State 4, in the nomenclature of Chance, and Williams (72). In the presence of pyruvate, the elevated levels of reduced pyridine nucleotides in the mitochondria as a result of the high activity of pyruvic oxidase is much greater than the oxidation of pyridine nucleotide in the cytoplasm, as shown by an increase of the tissue fluorescence. The present studies give further evidence of the compartmentation of intracellular reduced pyridine nucleotides in an intact organ (38, 73–75).

It has previously been shown that exogenous fuels such as acetoacetate, fatty acids, pyruvate, or acetate depress the utilization of the endogenous fuel reserves when added to the perfusates in high concentrations (1, 10, 76). In the present experiments, the addition of acetate to hearts perfused with glucose spared the utilization of 18 mg of lipid per g of heart, dry weight, per hour. If this lipid was composed entirely of triglyceride, and reutilization of glycerol negligible (77), release of glycerol into the perfusate should amount to at least 25 μmoles per g (dry weight) per hour in the absence of acetate. The observed glycerol release was only one-sixth this quantity, suggesting that phospholipid may comprise a large fraction of the lipid oxidized (78), or that esterification of glycerol to α-glycero-P was appreciable. Glycerol release was not influenced by the addition of acetate, but there was an increased incorporation of counts from ^{14}C-glucose into the glycerol moiety of the heart lipid, indicating an increased rate of esterification of intracellular fatty acids. A similar cycle of triglyceride hydrolysis and re-esterification is well established in adipose tissue (79), but in cardiac muscle it would appear to have a very low activity since the incorporation of isotope from ^{14}C-glucose into lipid represented at most only 2% of the count decrease from the medium.

Exogenous fuels probably inhibit the oxidation of endogenous lipid by a mechanism involving competition for cofactors, particularly CoA. Competition for CoA is also likely to be a major factor determining the rates of oxidation of different fuels when supplied in combination to the perfused rat heart (10). Similar considerations may account for the much greater accumulation of α-ketoglutarate in rat hearts perfused with pyruvate than those perfused with acetate. However, a discussion of the significance of the changes in the total amount of these intermediates found in whole tissue extracts must take into account the possibility of compartmentation. The presence of cytoplasmic malic enzyme and malic dehydrogenase allows the synthesis of malate and oxaloacetate from pyruvate in the cytoplasm, particularly at high pyruvate concentrations. Distribution of malate and oxaloacetate between the cytoplasmic and mitochondrial compartments may explain the poor agreement between values calculated for the cytoplasmic potential of the NAD:NADH system from the ratios of α-glycero-P to dihydroxyacetone-P and malate to oxaloacetate in the presence of pyruvate (Table VI).

Synthesis of citrate may also occur in the cytoplasm in addition to normal synthesis by the reactions of the citric acid cycle, as suggested previously (61). Citrate synthesis in the cytoplasm involves reductive carboxylation of α-ketoglutarate by NADP-linked isocitric dehydrogenase. The reducing power necessary for the carboxylations of malate and α-ketoglutarate could be provided by energy-linked transhydrogenation from NADH to NADP (80, 81). Preparations of heart muscle mitochondria rapidly catalyze this reaction under suitable conditions, and its operation in the presence of high pyruvate concentrations is indicated by the elevated tissue levels of NADPH. Possibly the phosphate potential of the cell is also increased, as shown by the increased levels of creatine phosphate. This would provide a further driving force for the transhydrogenation reaction in addition to the increased levels of NADH. The low activity of the citrate cleavage enzyme in heart (82) in combination with the poor permeability of the mitochondrial membrane to citrate may impose limits on the rate of removal of citrate from the cytoplasmic compartment.

With acetate as the major metabolic fuel, on the other hand, the decreased levels of oxaloacetate, and increased levels of acetyl-CoA (83) suggest that the relatively small accumulation of citrate is a direct result of the increased availability of acetyl-CoA. Depletion of oxaloacetate in the presence of acetate is buffered to some extent by glutamic-oxaloacetate transamination, as shown by the disappearance of aspartate and the appearance of an equivalent amount of glutamate. Glutamine and asparagine levels, on the other hand, did not change. In the presence of pyruvate, glutamate levels decreased by 50%, presumably because of transamination between glutamate and pyruvate since aspartate levels did not change appreciably. It is of interest that despite large changes in the concentration of glutamate, aspartate, and their respective keto acids, the K_{app} (59) for glutamic-oxaloacetate transaminase remained close to the equilibrium constant.

The present study illustrates that although different metabolic substrates produce large changes in the levels of many intermediates, the homeostatic mechanisms are so finely balanced by means of multiple control feed-backs that myocardial function, measured in terms of the total content of high energy phosphate compounds, oxygen consumption, and contractility,[1] is little affected. Further work, particularly on cell fractions, is needed to understand some of the finer control phenomena, especially the interrelationships between the activity of the citric acid cycle and metabolism in the cytoplasm.

SUMMARY

1. ^{14}C-Glucose has been used to study the pathways of glucose metabolism in the perfused rat heart in the presence and absence of insulin after the addition of acetate or pyruvate as alternative respiratory fuels. Changes in the tissue levels of glycolytic intermediates, adenine nucleotides, pyridine nucleotides, and most of the citric acid cycle intermediates have also been measured.

2. Acetate or pyruvate had similar effects on the over-all metabolism of glucose. Glycolytic flux was decreased in both the presence and absence of insulin, glucose oxidation was greatly decreased, and the conversion of glucose to glycogen and lactate was promoted. Glucose phosphorylation was decreased in the presence but not in the absence of insulin.

3. Expression of the results in terms of mass action ratios for each of the glycolytic reactions shows that hexokinase, phosphofructokinase, glyceraldehyde phosphate dehydrogenase, and pyruvic kinase are far displaced from equilibrium, while the other enzymic steps of glycolysis are maintained either at equilibrium or fairly close to equilibrium. In different metabolic situations, glycolytic flux may be affected by those steps which are far displaced from equilibrium.

4. Increased levels of the hexose monophosphates, and de-

[1] J. R. Williamson, unpublished observations.

sed levels of the other glycolytic intermediates between fructose 1,6-diphosphate and pyruvate after the addition of 10 mM acetate indicate that glycolytic flux was decreased by inhibition of phosphofructokinase. After the addition of 10 mM pyruvate, fructose 1,6-diphosphate, and triose-phosphate accumulated as a result of an inhibition of either glyceraldehyde phosphate dehydrogenase or phosphoglyceric kinase. Arguments are presented suggesting control at the former step by product inhibition.

5. Acetate had little effect on the total content of oxidized or reduced di- or triphosphopyridine nucleotides, but slightly decreased the ratio of nicotinamide adenine dinucleotide (NAD) to its reduced form (NADH) in the cytoplasm, as estimated from the ratios of lactate to pyruvate and α-glycerophosphate to dihydroxyacetone phosphate. Pyruvate increased the total content of NADH and reduced nicotinamide adenine dinucleotide phosphate as shown both by an increase of fluorescence in the intact heart, and by tissue analyses, but the ratio of NAD to NADH in the cytoplasm was greatly increased. These results demonstrate compartmentation of pyridine nucleotides between cytoplasm and mitochondria in the intact cell.

6. After the addition of acetate, citrate was the only intermediate of the citric acid cycle which increased greatly in amount, while oxaloacetate levels decreased. After the addition of pyruvate, the levels of citrate, α-ketoglutarate, malate, and oxaloacetate were all greatly elevated. The glutamic-oxaloacetate transaminase reaction as measured from the total contents the reactants in the tissue remained close to equilibrium.

7. Changes in the concentrations of the adenine nucleotides were insufficient to account for the inhibition of phosphofructokinase, but the results are consistent with control at this step being mediated by citrate.

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THE PROTECTIVE EFFECT OF SOME SODIUM COMPOUNDS AGAINST AMMONIA INTOXICATION IN THE AVIAN SPECIES*

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Abstract—1. The possible protective effect of some sodium salts of metabolizable acids against ammonia intoxication in chicks (*Gallus domesticus*) has been investigated.

2. The following compounds were shown to exert a significant protective effect against ammonia intoxication in the chick when administered intraperitoneally 1 hr prior to the ammonia challenge: 2, 3 and 4 m-moles/kg sodium acetate; 3 m-moles/kg sodium propionate; 3 m-moles/kg sodium butyrate; 3 m-moles/kg sodium bicarbonate; and 3 m-moles/kg potassium acetate. These compounds did not have a comparable effect in mice (*Mus musculus*).

3. Evidence is presented which indicates that the sodium ion of the metabolizable sodium salts exerts its effect by enhancing uric acid transport or excretion.

INTRODUCTION

SEVERAL studies have demonstrated that under certain conditions arginine exerts a distinct protective effect in preventing experimental amino acid and ammonia intoxication in mammals (Greenstein *et al.*, 1956; Gullino *et al.*, 1956; Najarian *et al.*, 1958; Salvatore & Bocchini, 1961; Manning *et al.*, 1964; Grossi *et al.*, 1967). An appropriate dose of an ornithine–aspartate mixture has also been reported to exert a similar effect in rats and dogs (Salvatore *et al.*, 1964; Grossi *et al.*, 1967). The mechanism of these protective agents has been consistent with an enhancement of the Krebs–Henseleit urea cycle. Potassium and magnesium salts of aspartic acid have also been reported to protect rats against ammonia intoxication, but the mechanism of these agents has not been elucidated (Rosen *et al.*, 1963).

Glycine and arginine have been shown to partially overcome the growth-depressing effects of high levels of single amino acids added to otherwise adequate diets in chicks (Snetsinger & Scott, 1961a). These workers were unable to show a protective effect of glycine or arginine in acute experimental amino acid or ammonia intoxication in chicks (Snetsinger & Scott, 1961b). However, recent work in our laboratory (Bloomfield *et al.*, 1969) has shown a protective effect of glycine and a glucose–glycine mixture on acute ammonia intoxication in chicks. The mechanism

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of the protective effect of glycine and the glucose–glycine mixture was shown to be involved in an enhanced biosynthesis of uric acid in the liver.

Stutz *et al.* (1967) have reported that salts of metabolizable acids such as potassium acetate, potassium citrate, potassium bicarbonate, sodium acetate and sodium bicarbonate, but not neutral salts, will stimulate growth in chicks. In the present study, the protective effects of various sodium salts of metabolizable acids on ammonium acetate intoxication in chicks are reported. Evidence is also presented that sodium acetate may act by enhancing the transport or excretion of uric acid in the chick.

MATERIALS AND METHODS

About 650 white leghorn (*Gallus domesticus*) male chicks were obtained from local hatcheries at 1 day of age and reared on a practical ration until the time of each study. Four hundred and eighty young female albino mice (*Mus musculus*), National Laboratory Animal Company, Crève Coeur, Missouri, with an average weight of 16·5 g, were also used in this investigation. All animals were fasted with water *ad lib.* for 24 hr prior to each study.

The animals were divided into four groups of ten animals per group for each compound indicated in Table 1. All compounds (reagent grade) were dissolved in distilled water so that approximately 1 ml of each solution was injected. The test compounds were injected intraperitoneally 60 min prior to an intraperitoneal injection of ammonium acetate which would produce approximately 70 per cent mortality. These doses were 12·5 and 15·0 m-moles/kg body wt. for the chicks and mice, respectively (Wilson *et al.*, 1968). The dose for the mice was slightly larger than that previously reported. The mortality ratio was recorded after exactly 60 min, at which time the surviving animals appeared normal.

For the serum uric acid studies, an intraperitoneal injection of 3 m-moles/kg body wt. of the test compounds were given to chicks 60 min prior to a LD_{50} intraperitoneal dose of ammonium acetate. The LD_{50} dose was 10·4 m-moles/kg body wt. (Wilson *et al.*, 1968). All chicks which survived the ammonium acetate injection were killed after 60 min and the blood was collected by decapitation. Blood was collected from the non-survivors immediately following the fatal extensor convulsion. Blood samples were combined from two chicks to obtain adequate serum for analysis. Serum uric acid was analyzed on a Technicon Auto Analyzer.

An isotope dilution method was used for the tissue uric acid determinations. An intraperitoneal injection of 3 m-moles/kg body wt. of sodium acetate was given 60 min prior to an intraperitoneal injection of 3 m-moles/kg body wt. of ammonium acetate. The chicks were killed with ether 60 min after the ammonia injection. The liver and right kidney were removed and placed in liquid nitrogen. The experiment was repeated and the total carcass was placed in liquid nitrogen. The frozen carcass was passed through a cooled meat grinder. The weighted ground carcass was then placed in a Waring Blendor, 5 μc of uric acid-2-^{14}C with a specific activity of 45·8 mc/m-mole was added, and the carcass homogenized with 5% $HClO_4$. The livers and kidneys were weighed, placed in a glass tissue homogenizer with a Teflon pestle, 1 μc of uric acid-2-^{14}C added and homogenized in 5% $HClO_4$. All homogenates were refrigerated at 0–5°C overnight to allow the added ^{14}C-uric acid to equilibrate with the uric acid in the sample. The filtrates were neutralized with KOH, the $KClO_4$ removed by centrifugation and the filtrates analyzed as previously described (Bloomfield *et al.*, 1969)

RESULTS

Table 1 summarizes the effect of the various compounds investigated on ammonia intoxication in chicks as compared to mice. Sodium acetate appeared to

be the best protective treatment ($P<0{\cdot}001$) and appeared to have an optimum concentration of 3 m-moles/kg body wt. in the chick. However, this dose was significantly ($P<0{\cdot}05$) more toxic in the mice. Sodium propionate, sodium butyrate and sodium bicarbonate were also significant protective treatments in chicks with no comparable effect in mice. However, sodium formate and sodium chloride had no significant effect in either species. Potassium acetate was almost as

TABLE 1—THE EFFECT OF VARIOUS COMPOUNDS ON AMMONIA TOXICITY IN CHICKS AS COMPARED TO MICE

Treatment	Chicks: mortality (%)*		Mice: mortality (%)*	
	Mean ± S.E.	*P* value	Mean ± S.E.	*P* value
Ammonium acetate (AA)†	70·0 ± 2·58	—	67·5 ± 4·80	—
Sodium formate (3 mm/kg)‡ + AA	57·5 ± 6·27	—	60·0 ± 7·03	—
Sodium acetate (2 mm/kg) + AA	37·5 ± 7·54	0·005	77·5 ± 4·80	—
Sodium acetate (3 mm/kg) + AA	17·5 ± 2·50	0·001	92·5 ± 4·80	0·05 §
Sodium acetate (4 mm/kg) + AA	50·0 ± 3·95	0·005	72·5 ± 8·83	—
Sodium propionate (3 mm/kg) + AA	37·5 ± 7·54	0·005	92·5 ± 4·80	0·05 §
Sodium butyrate (3 mm/kg) + AA	55·0 ± 5·00	0·05	95·0 ± 5·00	0·01 §
Acetic acid (3 mm/kg) + AA	90·0 ± 3·96	0·005 §	97·5 ± 2·50	0·005 §
Propionic acid (3 mm/kg) + AA	82·5 ± 4·80	0·05 §	100	0·001 §
Sodium bicarbonate (3 mm/kg) + AA	52·5 ± 4.80	0·01	87·5 ± 7·54	—
Sodium chloride (3 mm/kg) + AA	60·0 ± 3·96	—	72·5 ± 6·32	—
Potassium acetate (3 mm/kg) + AA	30·0 ± 10·80	0·005	75·0 ± 5·00	—

* Results of four groups of ten animals/group.

† The ammonium acetate doses were 12·5 and 15·0 m-moles/kg body wt. for chicks and mice, respectively.

‡ Dose as m-moles/kg body wt.

§ Significantly more toxic than the ammonium acetate alone.

effective ($P<0{\cdot}005$) in chicks as the sodium acetate treatment. Acetic and propionic acids were also studied in order to determine if the protective effect was due to the metabolizable anion. However, as can be seen, these treatments actually potentiated the ammonia intoxication. These data thus indicated that it is the cation, sodium or potassium, which is involved in the protective effect. It also appears that these cations need to be associated with a metabolizable anion.

From these data it is apparent that the protective effect of the sodium salts was specific for the chick and not a general physiological or metabolic phenomenon. Since uric acid is the major nitrogen excretion product in the chick, further studies were conducted to determine if these treatments were exerting their effect through uric acid synthesis or transport. The effect of pretreatment with sodium acetate on serum uric acid concentration is shown (Table 2). The concentration of uric acid in serum more than doubled following the administration of the LD_{50} of

ammonium acetate, but there was no significant difference between the sodium-acetate-pretreated chicks and the ammonium-acetate-treated controls.

TABLE 2—SERUM URIC ACID CONCENTRATIONS IN CHICKS FOLLOWING INTRAPERITONEAL INJECTION OF VARIOUS COMPOUNDS

Treatment	Dose (m-moles/kg body wt.)	Uric acid concentration (mean ± S.E. as mg/100 ml)	
		Survived*	Died
Controls	—	7·14 ± 0·58 (7)†	—
Ammonium acetate (AA)	10·4	18·60 ± 2·00 (6)‡	14·78 ± 1·73 (6)‡
Sodium acetate + AA	3 + 10·4	19·05 ± 3·06 (6)‡	23·60 ± 4·87 (2)
Sodium bicarbonate + AA	3 + 10·4	16·92 ± 2·15 (5)‡	16·98 ± 2·35 (5)‡
Potassium acetate + AA	3 + 10·4	15·75 ± 1·30 (4)‡	18·50 ± 1·99 (3)‡

* All animals which survived were killed after 60 min.

† Figures in parentheses indicate number of samples; each represents pooled serum from two chicks.

‡ Higher ($P < 0·001$) than the values for controls.

The results of the uric acid-^{14}C isotope dilution experiments are given in Table 3. There was no significant change in the size of the uric acid pool in the liver following either treatment. There was, however, a significant increase in the size of the uric acid pool in the kidney in the group treated with sodium acetate ($P < 0·01$) when compared to either the control or the ammonium acetate groups. The size of the uric acid pool in the total carcass was significantly higher ($P < 0·005$)

TABLE 3—TISSUE URIC ACID CONCENTRATIONS IN CHICKS FOLLOWING INTRAPERITONEAL INJECTION OF VARIOUS COMPOUNDS

Treatment	Dose (m-moles/kg body wt.)	Uric acid concentration (mean ± S.E.)		
		Liver (mg/g)	Kidney (mg/g)	Total carcass (mg/100 g)
Controls	--	0·180 ± 0·030 (10)*	0·200 ± 0·035 (10)	3·76 ± 0·42 (10)
Ammonium acetate (AA)	3·0	0·204 ± 0·045 (9)	0·221 ± 0·027 (10)	9·13 ± 1·42 (10)†
Sodium acetate + AA	3·0 + 3·0	0·180 ± 0·028 (10)	0·365 ± 0·041 (9)‡	10·10 ± 1·27 (10)§

* Figures in parentheses are number of samples per group.

† Higher ($P < 0·005$) than the values for controls.

‡ Higher ($P < 0·01$) than the values for controls and higher ($P < 0·01$) than the values for ammonium acetate.

§ Higher ($P < 0·001$) than the values for controls.

in the ammonium-acetate-treated group than in the controls and also higher ($P < 0{\cdot}001$) in the group treated with sodium acetate than in the controls. However, there was no significant difference between the sodium-acetate-treated group and the ammonium acetate group.

DISCUSSION

The results presented in this investigation show a significant protective effect of several sodium salts of metabolizable acids in chicks with no comparable effect in mice. Evidence is also presented which indicates that sodium acetate exerts its effect by enhancing transport or excretion of uric acid in the chick.

In our previous report (Wilson *et al.*, 1968) the intraperitoneal LD_{50} values for ammonium acetate were found to be essentially the same in mice and chicks. Based on the similar tolerance of ammonia by these species, it was suggested that the avian liver was as efficient as the mouse liver in detoxifying exogenous ammonia, thus indicating that purine or uric acid synthesis is quite important in detoxifying exogenous ammonia. The data in this report also support this conclusion in that the uric acid concentration in both serum and the total carcass increased two- to threefold following the injection of ammonium acetate. Additional evidence which supports this suggestion has recently been reported by Bloomfield *et al.* (1969), which demonstrated that glycine and a glucose–glycine mixture protected chicks against acute ammonia intoxication by an enhanced uric acid synthesis.

In the previous investigation (Bloomfield *et al.*, 1969) the uric acid content of liver, kidney and carcass was determined following pretreatment of the chicks with a glucose-glycine mixture. The data indicated an increase in uric acid concentration in the liver and kidney but no comparable increase in the total carcass. These data were interrupted to indicate an increase in uric acid synthesis. The incorporation of ^{15}N from $^{15}NH_3$ into uric acid in the total carcass was also shown to be enhanced in the glucose–glycine-treated chicks. In the present study, there was no change in the size of the uric acid pool in the liver, but there was a significant increase in the kidney pool size in the sodium-acetate-treated chicks. This shift in the uric acid pool size may indicate an enhanced transport of the uric acid from the liver to the kidney for excretion. This shift could possibly allow more uric acid to be synthesized in the liver, thus allowing more of the ammonia to be detoxified.

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Key Word Index—Ammonia intoxication; protection against intoxication; urea cycle; *Gallus domesticus*; *Mus musculus*.

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ACETIC ACID SENSITIVITY AS A CAUSE OF COLD URTICARIA

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THE subject of cold urticaria has been well documented since Blachez[1] first reported the condition in 1872 after Bourdon[2] had initially recognized it in 1866. Since then, many others have written on the subject, among them Lewis and Grant,[3] Horton and Brown,[4,5] Duke,[6] Bray,[7] Urbach,[8-10] Walzer,[11] Notier and Roth,[12] Sherman and Seebohm,[13] Rajka and Asboth,[14] Von Gerd,[15] Illig,[16] Herlitz,[17] and Sheldon, Mathews, and Lovell.[18] These authors have either presented cases describing the syndrome or discussed the probable immunologic mechanisms responsible for it, but no one has presented a case of cold urticaria which has been proved to be caused by a specific antigenic factor. To date, the cure of true allergic cold urticaria by means of allergic management has not been reported. It is the purpose of this discussion to describe a patient with a prolonged history of severe cold urticaria whose symptoms were related to the ingestion of acetic acid and were proved to be caused by a specific sensitivity to this acid. Since this patient had a remarkable clinical course and obtained a complete remission following allergic management, it seemed worth while to present the study of his case, especially with the thought in mind that new fields of investigation regarding causative mechanisms of physical allergies might be opened.

CASE PRESENTATION

P. A., a 35-year-old single male baker, was first seen on Jan. 26, 1954. He gave a four-year history of recurrent bouts of hives on the uncovered parts of the body following exposure to cold. The major areas involved were the head, face, ears, feet, and hands. He also complained of swelling in his throat following the ingestion of ice cream or very cold drinks. During recent years the hives became progressively worse, flaring up each fall at the onset of cold weather. The patient stated that he never had hives after swimming during the summer months. Antihistamines afforded him only temporary relief, and it was often necessary for him to remain in a warm room for over an hour before the swelling subsided. No symptoms suggesting dermographism were elicited. Hives had occurred intermittently all his life, and as a youngster he used to take Adrenalin under the tongue for relief of acute attacks. During the patient's childhood, citrus fruits, tomatoes, and certain brands of butter seemed to precipitate bouts of hives. He stated that he now ate tomatoes without difficulty, but still avoided citrus fruits. The patient had been a baker for the past ten years. He had lived at home with his parents in the lower half of a two-story house, where he had not been exposed to animal dander and had never followed dust precautions. Cottonseed flour was not used in the bakery, and no association between his symptoms and his work was noted. He used scented aftershave lotions and powders, and was exposed to average quantities of insecticides when the bakery was sprayed once a month by an exterminating service. The diet contained rather sizable quantities of chocolate, nuts, and spices, but the patient admitted a strong aversion to milk. He drank moderate amounts of beer and smoked six or seven cigars a day. Family history was completely negative for allergy as far as could be ascertained. Operations included a hemorrhoidectomy and a tonsillectomy.

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Complete physical examination was essentially negative except for an injected nasal mucous membrane and marked cloudiness on transillumination of the frontal and antral sinuses. Chest fluoroscopy, urinalysis, blood sugar, sedimentation rate, and Wassermann all gave normal results. The blood count showed a 5 per cent eosinophilia.

Scratch tests for dermographism were negative. When an ice cube was applied to his skin for three to five minutes, an urticarial lesion quickly formed in the exposed area. Blood was drawn and stored at refrigerator temperature for five weeks. Ice cube tests on sites in normal skin passively sensitized with the five-week-old serum were negative. The patient was seen approximately twice a week for further observation, and at each visit quickly showed a hive wherever an ice cube was placed on his skin.

Since patients with a physical allergy to cold usually get severe urticaria following swimming, the fact that our patient did not respond similarly was most puzzling. After exhaustive questioning, the patient finally remembered that he started to ingest very large quantities of sauerkraut at approximately the same time that his urticaria began four years previously. He was on a reducing diet and ate the sauerkraut because of its low caloric value. He remembered that he did not eat sauerkraut during the summer months, as it was unobtainable. He was therefore told to avoid sauerkraut and was observed for a change in his clinical condition. The following week the patient noticed a very definite diminution in his response to cold. When the ice cube was removed from the skin at this time, the hive did not form as quickly as had been previously observed, taking twenty to twenty-five minutes instead of three to five minutes.

During the next month, after institution of his elimination diet, he was quite comfortable but continued to keep a food diary. He noted that the ingestion of dill pickles, followed within an hour by a cold beverage, precipitated an edematous response in the mucous membrane of the hypopharynx. It was during a warm period in March that this reaction occurred. Since acetic acid is one of the main ingredients common to both sauerkraut and dill pickles, it was decided to investigate the possibility that sensitivity to this chemical compound was causing his allergic condition. With this in mind, the patient was advised to abstain from all foods which might contain acetic acid. He continued to be asymptomatic and, for the first time in four years, observed no hives following exposure to cold.

The sauerkraut was made from cranberries, cabbage, and salt, while the pickles contained vinegar, spices, thyme, dill, garlic, and sodium benzoate. On infrequent occasions when he had eaten plain cabbage or cranberries no unusual reactions had occurred. After obvious improvement had taken place during the first six weeks of the elimination diet, he again noted mild attacks of urticaria on exposure to cold shortly after adding sauerkraut or dill pickles to his diet. As soon as he avoided these foods, the hives subsided.

In order to eliminate the possibility that psychogenic factors may have influenced the results, special ingestion tests were performed. A thick carbohydrate placebo mixture with a strawberry-peppermint flavor was prepared with lemon juice added in an attempt to simulate an acid flavor. Because of the warm weather at this time, the only way the reaction could be reproduced was to have the patient consume some chilled material following ingestion of the test mixture. He was given about 15 ml. of the placebo mixture on two different occasions, following which he immediately ate some ice cream and noted no unusual reaction. He was then given 15 ml. of the placebo mixture with the addition of about 2 ml. of 2 per cent acetic acid. He again ate some ice cream and within fifteen minutes felt his throat begin to swell. The acetic acid had been obtained from ordinary vinegar. This 2 per cent solution was then diluted 1:1,000, 1:100, and 1:10 for skin testing purposes. Direct skin testing with these dilutions produced no greater reaction in the patient than was obtained on testing control subjects. It was observed that, in spite of his previous supposed sensitivity to citrus fruits, he could now ingest lemon juice, such as was used in the placebo mixture, without any untoward effects, indicating that probably he was no longer sensitive to lemon or other citrus fruits.

After a six-month lapse in observation, the patient has recently been restudied. He had been following the elimination diet very carefully. The ice cube test was now complete-

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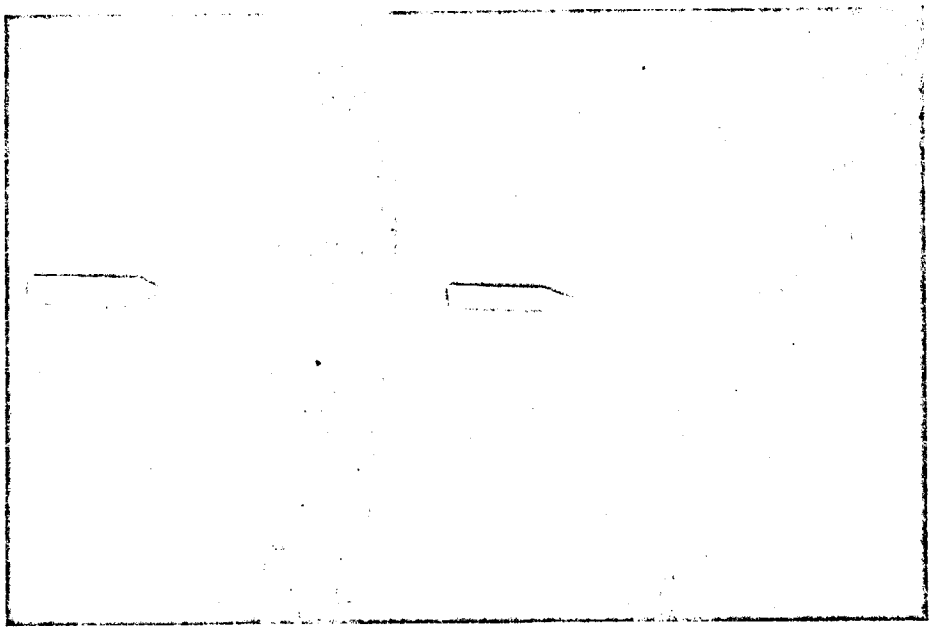
negative, even after application to the skin of the forearm for a period of fifteen minutes. He was able to take pans out of a freezer at 10° F. in the bakery with his bare hands.

Because of the patient's previous history suggesting a possible sensitivity to citric acid and the fact that both lactic acid and acetic acid are found in large quantities in sauerkraut, it was decided to skin test him with chemically pure acetic, lactic, and citric acids. After no signs of cold urticaria had appeared for many months, skin tests on Dec. 7, 1954, with these .1 per cent chemically pure acids were negative. Tests done with .01 per cent acid solutions, followed by application of an ice cube over the skin test area for fifteen minutes, were also negative. When the intradermal tests were repeated, however, with .1 per cent acids, followed by application of an ice cube to the test sites for fifteen minutes, a definite urticarial response was obtained over the acetic acid site which gradually swelled into a wheal larger than a half dollar (4 cm. in diameter) during the next half-hour. No similar reaction was observed over the lactic acid or citric acid sites.



A. B.

Fig. 1.—A, Skin test with .1 per cent chemically pure lactic acid twenty-five minutes after ice cube was placed over test site. B, Skin test with .1 per cent chemically pure acetic acid twenty-five minutes after ice cube was placed over test site.

The same reaction was repeated on three separate occasions within a period of one week and each time, after removal of the ice cube from the acetic acid test site, the wheals began to form within five minutes and reached their maximum size five to ten minutes later (twenty to twenty-five minutes following the original contact of the ice with the acetic acid test site). Application of ice to the skin concomitantly for fifteen minutes in an area not previously skin tested, or in an area where a skin test with a .1 per cent lactic acid test had been performed, produced no urticarial response in either site (Fig. 1). The patient further stated that the lesion produced in the acetic acid test site definitely itched and felt like a typical hive. Placing an ice cube for fifteen minutes over a skin test with .1 per cent chemically pure acetic acid on several control subjects (nonallergic persons) gave completely negative reactions. Unfortunately, during the preliminary passive transfer studies of the

patient's serum, a skin test with acetic acid followed by an ice cube over the test site was not done. When this procedure was eventually carried out on the original serum after it had been refrigerated for ten months the test was negative.

Six weeks after the positive direct skin tests had been obtained, serum was withdrawn for additional passive transfer studies. Nonallergic persons were then sensitized with four-day-old filtered serum and were tested by placing an ice cube over an area previously injected with .1 per cent chemically pure acetic acid. No positive reactions were obtained. A few days later nonallergic subjects were sensitized with unfiltered serum four hours after it had been withdrawn. Skin testing of these sites with acetic acid plus an ice cube over the test site produced no positive responses, regardless of whether the tests were performed immediately or twenty-four hours following sensitization. Direct skin testing of the patient at this time with acetic acid in the presence of cold also produced a negative reaction. (See Table I for summation of testing results.)

TABLE I. SUMMATION OF TESTING RESULTS

DIRECT TESTING			INDIRECT TESTING		
DATE	TESTING MATERIAL	RESULTS	DATE	TESTING MATERIAL	RESULTS
3/20/54	Acetic acid .01%	Negative	2/ 1/54	Serum drawn	
3/20/54	Acetic acid .1%	Negative	3/ 8/54	Ice cube test on 2/1/54 serum	Negative
3/20/54	Ice cube test	Positive	12/ 7/54	.1% C.P.A.A. and ice cube over test site on 2/1/54 serum	Negative
12/ 7/54	.1% C.P.A.A.*	Negative	1/14/55	Serum drawn	
12/ 7/54	.1% C.P.A.A. and ice cube over test site	Positive	1/18/55	.1% C.P.A.A. and ice cube over test site on 1/14/55 serum	Negative
12/ 7/54	Plain ice cube test	Negative	1/24/55	Serum drawn	
12/ 7/54	.1% C.P.L.A.†	Negative	1/24/55	Tests performed on unfiltered serum with .1% C.P.A.A. and ice cube over test site 4 hours following withdrawal on 1/24/55	Negative
12/ 7/54	.1% C.P.L.A. and ice cube over test site	Negative	1/25/55	Tests performed on unfiltered serum with .1% C.P.A.A. and ice cube over test site 24 hours following withdrawal on 1/24/55	Negative
12/ 7/54	.1% C.P.C.A.‡	Negative			
12/ 7/54	.1% C.P.C.A. and ice cube over test site	Negative			
12/ 9/54	.1% C.P.A.A. and ice cube over test site	Positive			
12/ 9/54	.1% C.P.L.A. and ice cube over test site	Negative			
12/12/54	.1% C.P.A.A. and ice cube over test site	Positive			
12/12/54	.1% C.P.L.A. and ice cube over test site	Negative			
1/24/55	Plain ice cube test	Negative			
	.1% C.P.A.A. and ice cube over test site	Negative			

*C.P.A.A. chemically pure acetic acid.
†C.P.L.A. chemically pure lactic acid.
‡C.P.C.A. chemically pure citric acid.

He was then instructed to eat foods made with acetic acid to see if the hives would recur. During the next three months, he continued to ingest sauerkraut frequently without developing hives or any symptoms suggesting a mucous membrane hypersensitivity to cold.

The fact that we were not able to passively transfer the patient's reaction to acetic acid in the presence of cold means that if reagins were present they probably were produced for only a short time and that, if the studies could have been done at exactly the same time the direct reactions were obtained, reagins might have been demonstrated. Knowing that the reagenic sensitivity, which does occasionally occur in drug sensitivities, is usually of a transient nature, it is not surprising that our patient may have had a similar response.

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The only previously recorded case of a patient who was thought to have cold urticaria from a specific chemical sensitivity was described by Herlitz.[17] He discussed a 15-month-old girl who appeared to have urticarial-like lesions on the exposed parts of her body after being out in cold weather. She was being treated at the time with Tulo tablets (throat lozenges containing menthol). The skin eruption had been present for only a few days and appeared to be directly related to the ingestion of these tablets, since the reaction stopped shortly after she was taken off the lozenges and reappeared following reinstitution of this medication. A confusing aspect of the case report, however, was the fact that when the patient was again given the lozenges, her skin lesions recurred following exposure to cold weather, but the ice cube test was negative. It is difficult to understand how one with active cold urticaria can have a negative ice cube test, especially when it was previously positive.

Abramson[19] reported a case of nonallergic cold urticaria which responded well to psychiatric therapy. There was no evidence in the patient's personal or family history to indicate that it was an allergic problem.

Urbach, Linneweh, and Greenberg[20] described a patient who developed urticaria after the ingestion of pork sausage which contained large amounts of pork blood. Following repeated exposures to the material, he eventually developed cold urticaria, as the lesions would only occur on areas just previously bathed with cold water. When given plain pork sausage which did not contain any pork blood, the reaction to cold did not take place. Since the patient signed out of the hospital prematurely, no farther investigative studies could be conducted to determine what factor in the pork blood was responsible for the reaction.

Two additional cases of cold urticaria which responded well to treatment have also been reported by Urbach.[8, 9] One was a patient with a localized tooth infection whose urticaria cleared following removal of the tooth. The other was a 25-year-old woman with an intermittent twelve-year history of cold urticaria following the onset of her menses at age 13. The only periods free of urticaria during this time were while she was pregnant. The pelvic findings were normal, and the urticaria ceased after she received injections of posterior pituitary hormone combined with x-ray therapy to the pituitary gland.

Because of the belief that acetic acid is a normal constituent of body metabolism, it may be questioned whether hypersensitivity to acetic acid can occur. Personal discussion of this point with Dr. W. W. Westerfeld,[21] professor of biological chemistry at New York State College of Medicine, Syracuse, New York, has revealed the fact that currently it is believed that acetic acid is not a normal intermediate in body metabolism. As early as 1933, Himwich and his group observed that no free acetic acid could be found in the blood following the metabolism of ethyl alcohol. Later, in 1939, Friedemann and Kmieciak[22] confirmed the work of Himwich and his associates,[22] adding further evidence to show that free acetic acid is not a normal breakdown product of body metabolism. In so far as is known, acetic acid is not present in the free state in any measurable quantity but is present in combination with a coenzyme,

CoA, as acetyl CoA. Because free acetic acid is not a normal product of body metabolism, it is possible that hypersensitivity to this compound could occur in human beings.

It would appear that the patient discussed in this article had a specific chemical sensitivity which produced the cold urticaria and that, following the elimination of this chemical from his diet, the allergic activity quickly subsided. The findings suggest that, in the presence of cold, either (1) the acetic acid acted as a hapten to form a complex antigen responsible for the allergic reaction or (2) the cold acted as a more suitable medium to permit the acetic acid hypersensitivity to take place. In this respect, the cold could have been a catalyst in the allergic reaction.

We believe that these findings are also significant because a new technique of testing for drug sensitivity may have been demonstrated. This type of testing may be especially valuable in the study of patients with physical allergies due to chemical sensitivities.

CONCLUSION

1. The case history has been presented of a patient who has apparently been cured of recurrent cold urticaria and mucous membrane hypersensitivity to cold of four years duration by avoidance of foods containing acetic acid.

2. After diet elimination, ingestion of a placebo mixture followed by ice cream produced no mucous membrane response. When a minute quantity of acetic acid was added to this mixture and the ingestion was followed by ice cream, pharyngeal edema quickly developed.

3. Direct skin testing with .1 per cent chemically pure acetic acid was negative, but when an ice cube was immediately placed over the test site for fifteen minutes an urticarial reaction resulted. Ice cube tests performed concomitantly over untested skin or over an area tested with .1 per cent chemically pure lactic or citric acid were negative. Similar testing with .1 per cent chemically pure acetic acid plus an ice cube over the test site in normal control subjects gave negative reactions.

4. A new technique for skin testing for drug sensitivities in patients suffering from physical allergies has been suggested.

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608 E. GENESEE ST.

THE ACUTE ORAL TOXICITY OF ACETIC, CHLORACETIC, DICHLORACETIC AND TRICHLORACETIC ACIDS*

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THE PROPOSED use of chloracetic acid as a preservative for foods and beverages has raised the question of the toxicity of this substance. Since at one time or another acetic, dichloracetic and trichloracetic acids have also been used or proposed for use as antiseptics or preservatives, it was decided that the relative acute toxicities of these substances should be determined; and since the time required for chronic toxicity studies is long, these relative acute toxicities are being presented in order that those interested may have this information available. Except in the case of acetic acid there are very few published data on the toxicity of these substances. Lamb and Evvard (1) fed as much as 500 cc. of a 1 N solution of acetic acid daily to each of two pigs for 150 days and observed no significant effects. Schibkow (2) has reported that if the vapors of acetic acid are inhaled, death is caused in white mice during the experiment or a short time after its conclusion, and he has stated that the death was due to an intoxication but does not explain the intoxication. Dreyfus (3) induced death in rabbits in a few hours by rectal administration of 40 cc. of a 3% solution of acetic acid. Sollmann (4) gave acetic acid to rats in their drinking water in concentrations as high as 0.5%. The animals on 0.25% were not affected but the growth rate was inhibited in the rats on 0.5%. This latter group, however, were receiving from 310 to 710 mg. of the acid per day and survived for the experimental period of 9 weeks. Busacca (5) reported that the chlormethyl ester of acetic acid was very toxic and proposed its use in chemical warfare. Chabrol, Charonnat, Maximin and Waitz (6) reported that chloracetic acid has a cholagogic action in doses of 0.6 gm., increasing the bile flow two to four times. They also reported that trichloracetic acid increased the bile flow by two times and raised the concentrations of solids in the bile from 2.7 to 3.15 gm. per 100 cc. Genevois and Brisou (7) have stated that mixtures of the monohalogenated acetic acids, consisting chiefly of chloracetic acid, are nontoxic in comparison with each acid separately. Tetsumoto (8) reported that trichloracetic acid was the most effective of the chlorinated fatty acids against pathogenic bacteria *B. typhosus* and *V. cholerae*.

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Through personal communication with Dr. Chauncey D. Leake, we have had the privilege of reading a paper on the chronic toxicity of chloracetic acid which he is to publish in the University of California Publications in Pharmacology.

PROCEDURE

Since it was desired to determine the relative toxicities of the acid radicals and not their toxicities as acids, that is in terms of hydrogen ions, and since the ionization constants of the acids under investigation are acetic 1.86×10^{-5}; chloracetic, 1.5×10^{-3}; dichloracetic, 5×10^{-2}; and trichloracetic, 2×10^{-1}, the acids before administration were adjusted with sodium hydroxide to a range of pH between 6 and 7. The concentrations of the solutions used were such that the animals received approximately 0.01 cc. of solution per gram of body weight.

The mice used were young adult Albinos of a heterogeneous stock with the sexes equally distributed. The rats used were predominantly males which were raised in this laboratory and which ranged in weight from 150 to 250 gm. and in age from 70 to 100 days. The guinea pigs were stock animals, all males, and ranged in weight from 450 to 600 gm. The solutions were administered to the rats and guinea pigs orally by means of a soft rubber catheter attached to a syringe. They were similarly administered to the mice, except that a straight metal catheter was used. The rats and guinea pigs were fasted 18 hours prior to administration of the solutions. Immediately following the treatment the animals were observed for the 6 days and any animals which survived were not used in subsequent experiments. The dosage-mortality curves are constructed for each substance according to the methods of Bliss (9) and according to the example described by Laug, Calvery, Morris and Woodard (10). Animals receiving the acetic, dichloracetic, and trichloracetic acids quickly passed into a state of narcosis or seminarcosis, and within 36 hours either recovered completely or died without coming out of the narcosis. Animals receiving the chloracetic acid usually exhibited no immediate striking symptoms, but became apathetic and rapidly lost weight. When death occurred it was within 1 to 3 days, and those animals which survived for this period completely recovered.

TABLE 1

LD50, LIMITS OF LD50 WITH A CERTAINTY OF 95% (BOTH EXPRESSED AS GMS. OF ACID PER KG. BODY WEIGHT), AND SLOPE OF DOSE-RESPONSE CURVE "b"

NAME OF ACID	RATS				MICE				GUINEA PIGS			
	LD50	1.96σ (P = 0.95)		Slope "b"	LD50	1.96σ (P = 0.95)		Slope "b"	LD50	1.96σ (P = 0.95)		Slope "b"
		Lower	Upper			Lower	Upper			Lower	Upper	
Acetic	3.31	3.00	3.66	7.71	4.96	4.43	5.55	7.53				
Chloracetic	0.0762	0.0707	0.0822	8.09	0.255	0.196	0.334	3.27	0.0793	0.0718	0.0886	9.72
Dichloracetic	4.48	4.29	4.69	27.00	5.52	3.81	8.00	4.85				
Trichloracetic	3.32	3.16	3.48	20.97	4.97	4.70	5.26	11.87				

The results show that dichloracetic

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TABLE 2

DOSES OF ACIDS ADMINISTERED AND NUMBER OF FATALITIES RESULTING FROM EACH DOSE

(The dose in each case is expressed in gms. of the acid per kg. of body weight, while actually the sodium salt of the acid was used)

SUBSTANCE	MICE		RATS	
	Dose	Mortality ratio	Dose	Mortality ratio
	gm./kg.		*gm./kg.*	
Acetic acid	2.8	0/10	2.239	1/10
	3.548	2/10	2.512	0/10
	4.2	3/10	2.859	3/5
	5.012	4/10	3.1	5/10
	5.6	7/10	3.5	6/10
	6.31	8/10	4.0	7/10
			4.467	8/10
Chloracetic acid	0.0794	1/10	0.0375	0/5
	0.100	2/10	0.060	4/20
	0.1259	1/10	0.070	5/10
	0.1413	0/10	0.075	2/5
	0.150	1/10	0.080	12/20
	0.1585	2/10	0.090	10/20
	0.1778	4/10	0.100	15/15
	0.1995	6/10	0.120	10/10
	0.2239	4/10	0.150	10/10
	0.2512	5/10		
	0.2818	5/10		
	0.3162	8/10		
Dichloracetic acid	3.00	2/10	2.20	0/10
	3.162	1/10	2.50	0/10
	4.00	2/10	2.80	0/10
	5.012	3/10	3.20	0/10
	5.623	4/10	3.60	0/10
	6.310	7/10	4.00	1/10
	7.943	8/10	4.40	4/10
	8.913	9/10	4.80	8/10
Trichloracetic acid	3.162	0/10	2.594	0/5
	3.981	1/10	3.00	3/10
	4.217	1/10	3.153	1/5
	4.467	2/10	3.40	5/10
	4.50	7/10	3.80	9/10
	4.732	5/10	3.991	5/5
	5.012	3/10	4.20	10/10
	5.309	4/10	4.6	10/10
	5.623	7/10		
	6.00	10/10		

TABLE 2—*Concluded*

SUBSTANCE	GUINEA PIGS		RABBITS	
	Dose	Mortality ratio	Dose	Mortality ratio
	gm./kg.		*gm./kg.*	
Chloracetic acid	0.060	1/10	0.050	0/5
	0.070	4/10	0.065	0/4
	0.080	3/10	0.075	1/4
	0.090	8/10	0.080	0/3
			0.085	0/10
			0.090	5/10
			0.095	4/9
			0.100	1/1
			0.125	1/1
			0.150	1/1

acid is the least toxic, acetic acid and trichloracetic acid only slightly more toxic than dichloracetic acid, and chloracetic acid much more toxic than any of the other three.

The LD50 for each substance, the range in dose in which 19 times in 20 the LD50 would fall, also for each substance, and the slope of the curve "b" are given in table 1. From these data it is evident that the LD50 for chloracetic acid is only about $\frac{1}{40}$ to $\frac{1}{25}$ of that of the other acids. A tabulation of the doses and the number of animals killed at each dose is presented in table 2. The relative positions and shapes of the dosage mortality curves are shown in the chart.

SUMMARY

1. The relative acute toxicities have been determined for acetic, chloracetic, dichloracetic and trichloracetic acids.

2. The LD50's (in grams per kilogram) are: acetic acid, for rats 3.31, for mice 4.96; chloracetic acid, for rats 0.076, for mice 0.255, for guinea pigs, 0.080; dichloracetic acid, for rats 4.48, for mice 5.52; trichloracetic acid, for rats 3.32, for mice 4.97.

3. The limits of the LD50 with a

certainty of 95% (1.96 σ (P = 0.95)) and the slope of the dose response curve "b" have been determined.

4. These experiments show that the acute oral toxicities, as measured by the LD50, of acetic, dichloracetic, and trichloracetic acid are of the same order of magnitude, while the toxicity of chloracetic acid for mice is approximately 25 times and for rats, approximately 40 times those of the other three acids.

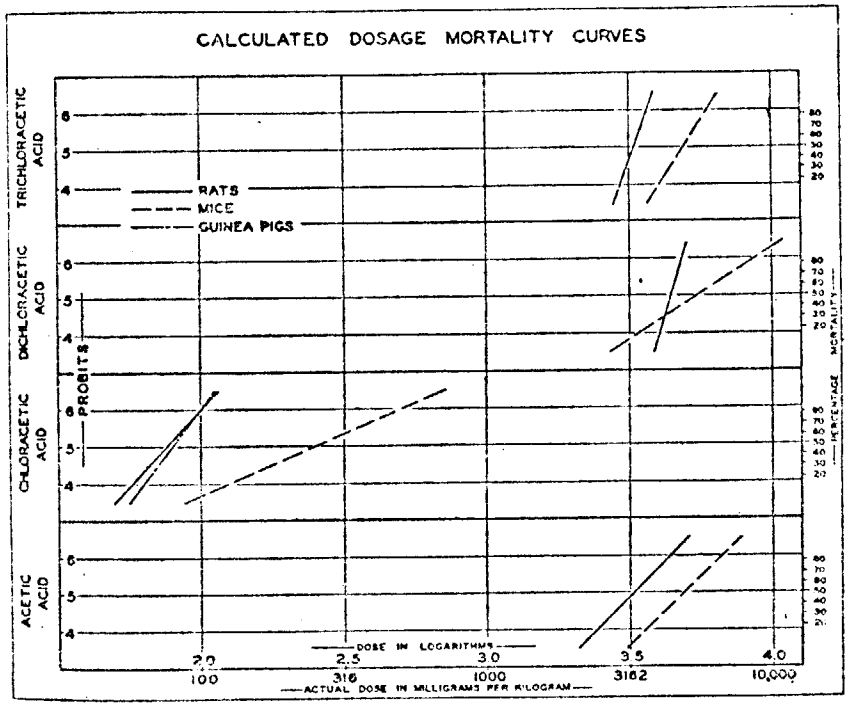

FIG. 1

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Nutritional Contribution of Volatile Fatty Acids from the Cecum of Rats[1]

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ABSTRACT The contribution of cecal acetic, propionic and butyric acids to rats' energy metabolism amounted to 4.7% of the caloric intake. This was determined by measuring rates of disappearance of these volatile fatty acids (VFA) from the cecum of rats killed at various intervals after the last meal. Further support indicating that VFA are energy sources for rats came from the appearance of $^{14}CO_2$ in the breath when radioactive VFA were placed in their cecum.

Approximately 70% of the metabolizable energy of ruminants has been reported to be supplied by volatile fatty acids (VFA) produced in the rumen (1, 2). However, the quantity of VFA derived by monogastric animals from their alimentary tract including the cecum is not well documented. Recently, langur monkeys and porcupines have been found to produce VFA in the stomach and cecum, respectively (3,4). In langurs, gastric VFA reportedly contributed energy in excess of that required for maintenance whereas in porcupines the cecal VFA supplied from 16 to 33% of their requirement.

Even though very little information is available concerning the nutritional contribution of alimentary VFA in monogastric animals, at least nine different species of monogastric animals have VFA in their cecum (table 1). The concentrations of these acids are as high as, if not higher than, those found in the rumen. In rats VFA are utilized for various functions including the formation of glycogen (10, 11). When radioactive carboxyl-labeled acetic, propionic and butyric acids were fed together with glucose to fasted rats, 35.8, 24.0 and 45.6%, respectively, of the dietary radioactivity appeared in the respiratory CO_2 in 2 hours suggesting that VFA are metabolized. Furthermore, acetic and butyric acids in the form of triglycerides and making up about 50% of the gross dietary energy of rats supported growth almost to the same degree as control diets. However, propionic acid in triglyceride form caused death.[2] In chickens, calcium salts of these acids when fed (2.5% of diet) were not recovered in gut suggesting that these acids are utilized (12).

Evidence suggests not only the utilization of VFA from the alimentary tract by monogastric animals but that the quantity of these acids in the cecum of rats can be increased by the addition of cellulose to the diets (9). These observations thus also suggest that cecal VFA can be an important source of energy to the monogastric animals especially if diets contain high proportions of cellulose. The present studies were therefore designed to determine the quantity of endogenous VFA disappearing from the cecum and to ascertain whether these acids are absorbed and metabolized to form respiratory carbon dioxide.

METHODS

Sixty 3-week-old male rats of the Sprague-Dawley strain were fed a laboratory grain diet[3] and water ad libitum for 6 weeks. After this period, they were allowed access to the grain diet for 2 hours each day from 9:00 to 11:00 AM. The rats were housed individually in suspended wire cages in a room maintained at 22° and 12 hours each of light and darkness. Of the 60 rats, 48 were chosen for this study based on their consistent body weight gain during the restricted feeding period. Twenty-four of these selected rats were killed three at a time starting immediately after the last meal (time zero) on day 35 of restricted feeding and at 2, 4, 8, 12, 16, 24 and 48 hours thereafter. The remaining 24 selected rats were killed according to a similar schedule except that the last meal was on day 42 of the restricted period. Only three rats could be handled adequately at each time to minimize further digestion and fermentation of digesta during the many manipulations needed to obtain the digesta quantitatively.

The rats were killed by decapitation and the ceca removed. Cecal digesta were removed and weighed. A known portion of the digesta was added to an equal weight of 0.7 N H_2SO_4 to stop bacterial fermentation. The acidified digesta were stored in a refrigerator for 2 days and then centrifuged to obtain supernatant. Concentrations of acetic, propionic and butyric acids in the supernatant were determined by gas-liquid chromatography using standard curves obtained from known quantities of these acids.[4] The total quantities of VFA were then calculated for each time interval after the last meal.

In a separate study, male rats of the Sprague-Dawley strain were fed the grain diet ad libitum. These rats weighed an average of 485 g at about 20 weeks of age and were used to determine whether radioactive VFA placed in the cecum were absorbed and metabolized. For this purpose, the rats without previous fasting were anesthetized with ether at 10:30 AM, and a 2-cm midventral incision was made to expose the cecum. Sodium salts of acetate-1-^{14}C, propionate-1-^{14}C and butyrate-1-^{14}C dis-

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[3] Percentage composition of the grain diet was: ground corn, 60.7; soybean meal (50% protein), 28.0; alfalfa meal (17% protein), 2.0; fish meal (12.5% protein), 2.5; dried whey (67% lactose), 2.5; limestone (38% Ca), 1.6; dicalcium phosphate (18.5% P, 22 to 25% Ca), 1.75; iodized salt, 0.5. Supplementary minerals and vitamins were added to provide per kg of diet: (in mg) Mn, 121; Fe, 95; Cu, 7; Zn, 4; I_2, 4; Co, 2; choline chloride, 400; Ca pantothenate, 6; riboflavin, 3; niacin, 33; menadione, 9; DL methionine, 500; (in μg) vitamin B_{12}, 7; (in IU) vitamin A, 8010; vitamin D_3, 750; vitamin E, 5.

TABLE 1

Concentrations of VFA in the ceca of several species of monogastric animals

Species	Approximate age or weight	Diet	Acetic	Propionic	Butyric	References
			mEq/g dry content			
Chicken	17 Weeks	Standard layer feed	0.42	0.21	0.007	Annison et al. (5)[1]
Guinea pig	Adult (?)	Standard commercial (?)	---- 0.38 acetic equivalent ----			Hagen and Robinson (6)
Hamster	Adult (?)	Standard commercial (?)	---------- present ----------			Hoover et al.[2]
Horse	580 kg	Grass, straw and marigolds	0.75	0.17	0.05	Elsden et al. (7)
Pig	4 Weeks	Bran	0.40	0.15	0.04	Elsden et al. (7)
Pig	84 kg	Barley, whey, bran	0.72	0.51	0.10	Friend et al. (8)
Pig	6 Weeks	Casein, soy, cerelose, cellulose and fat	0.31	0.14	0.04	Hendricks et al.[3]
Porcupine	10 kg	Natural wild vegetation	0.38	0.06	0.07	Johnson and McBee (4)[1]
Rabbit	Adult (?)	Bran, oats and marigolds	0.26	0.03	0.03	Elsden et al. (7)
Rat	Adult (?)	Bran, oats and hay	0.37	0.15	0.14	Elsden et al. (7)
Rat	487 g	Natural grain mix	0.50	0.07	0.15	Yang et al. (9)[1]

[1] Calculations for this table were made on the assumption that the cecal dry content was 15% of the wet contents for chicken, porcupine and rat.
[2] W. H. Hoover, C. L. Manninger and H. E. Sheperin. 1968. Observations pertaining to digestion in the golden hamster. J. Anim. Sci. 27: 1512 (abstract).
[3] D. Hendricks, E. R. Miller, D. E. Ullrey and M. G. Yang, unpublished data.

[4] The gas chromatograph used in the GLC method was F and M model 810 equipped with flame ionization detectors. The column used was 183 x 0.32 cm Teflon tubing packed with chromosorb W, A/W, DMCS, 80/100 mesh coated with 10% FFAP. The support and liquid phase were purchased from Varian Aerograph, Walnut Creek, California. The oven temperature was isothermal at 150°.

solved in water were injected directly into the exposed cecum. Four, three and three rats were used for acetate, propionate and butyrate, respectively. The radioactivity of each injected acid was 1.0 μCi in 0.5 ml water. The specific activities of the three acids were 40, 49 and 25 μCi μmole, respectively, for acetic, propionic and butyric acids. The high specific activities of these acids permitted an extremely small quantity to be added to the cecal content. Immediately after introducing the radioactive VFA the incision was closed with autoclips[5] and the rats were placed in collection chambers similar to the one described by Gunsalus et al. (13).

From preliminary studies, the first two of a series of three traps filled with 5, 3 and 5 ml hyamine hydroxide were found to effectively trap more than 99.5% of the expired $^{14}CO_2$. The third trap was a precautionary measure. This arrangement was followed in the present experiments. Every 15 minutes for 2.5 hours the two traps closest to the metabolic chamber were replaced with two new traps which allowed a close monitoring of the $^{14}CO_2$ respiration rates. Another reason was that when longer periods were used, $^{14}CO_2$ level increased in the third trap indicating washing out of CO_2 by expired and residual air supplied to the rats. Furthermore, there was a tendency for hyamine hydroxide to dry out when the periods were extended. The radioactivity in the trapped $^{14}CO_2$ was determined by channel ratio technique using a liquid scintillation detector counter.[6] POPOP[7] and PPO[8] in toluene were used as fluor.

RESULTS AND DISCUSSION

In all cases, the amount of VFA increased in the cecum from time zero to 2 hours after the last meal after which it decreased (figs. 1, 2, and 3, solid lines). Butyric acid was the only acid which was absent or in extremely small quantities in the digesta at 24 hours after the last meal whereas acetic and propionic acids were drastically reduced. The quantities of VFA between 0 and 4 hours after the last meal were comparable to those found in other studies with rats (9). Since time zero corresponded to the end of 2 hours of eating,

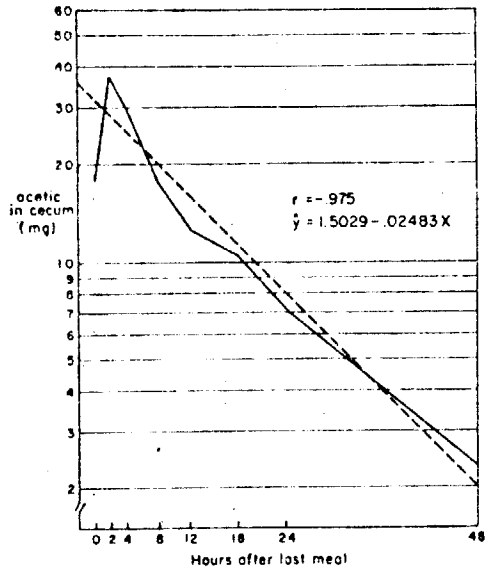

Fig. 1 Semilogarithmic plot and regression line (broken line) of amount of cecal acetic acid versus time after last meal which represent the disappearance of acetic acid from rats' ceca during the 48 hours following their last meal. The rats had previously been trained to eat their day's feed between 9:00 and 11:00 AM.

the VFA values at time zero were not included in determining regression lines (figs. 1, 2 and 3, broken lines). The regression equations were thus obtained for each acid using values starting at 2 hours after the last meal (14). Correlation coefficients (r values) greater than 0.97 were obtained for each of the VFA.

The regression lines in figures 1, 2 and 3 are typical curves often used to describe exponential functions. Thus, the rate of decrease at any time on the regression line is dependent on the quantity of VFA present at that particular time. These exponential functions are the same as those used to describe radioactivity decay or first-order reactions (15). The following equation was therefore adapted to describe the results of the present experiments:

$$Q_t = Q_0 e^{-kt},$$

where Q_t = quantity of VFA at any time, Q_0 = quantity of VFA at time zero which can be obtained by extrapolation from the regression lines, e = base of natural logarithms, t = time in hours, and k = a constant which is dependent on the half-life ($T_{1/2}$) or length of time in hours required to reduce the quantity of VFA at any point on the regression line to half its initial value. The constant k can be determined by dividing 0.693 by $T_{1/2}$, the half-life (15).

Total VFA disappearing over a 24-hour period is represented by the triangular area above the regression line within the boundaries set by 24 hours and the quantity of acid at time zero. This area is the total integrated decrease from time zero to time t which can be obtained by integrating the exponential function from 0 to time t as follows:

$$f = \int_0^t Q_0 e^{-kt}\,dt = \left[-Q_0 \frac{e^{-kt}}{k}\right]_0^t = -\frac{Q_0}{k}(e^{-kt} - 1)$$

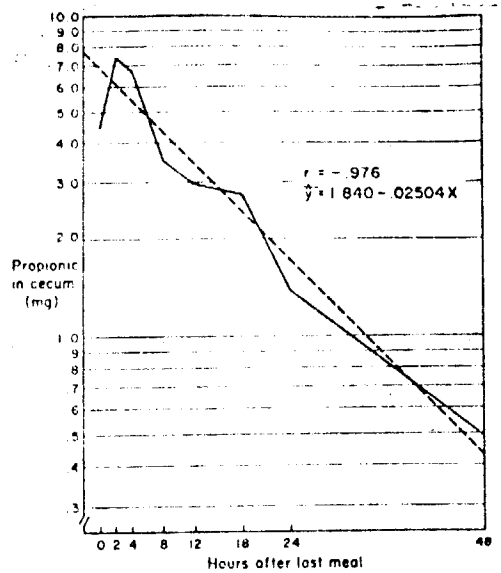

Fig. 2. Semilogarithmic plot and regression line (broken line) of quantity of cecal propionic acid versus time after last meal which represent the disappearance of propionic acid from rats' ceca during the 48 hours following their last meal. Further details given in caption for figure 1.

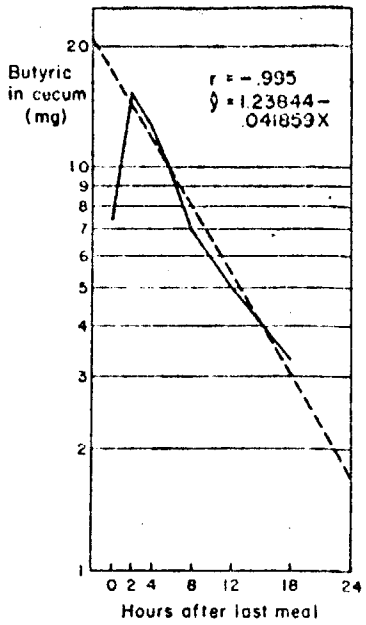

Fig. 3 Semilogarithmic plot and regression line (broken line) of quantity of cecal butyric acid versus time after last meal which represent the disappearance of butyric acid from rats' ceca during the 24 hours following their last meal. Further details given in caption for figure 1.

Q_0 and $T_{1/2}$ were estimated directly from the regression lines. The values for $T_{1/2}$ were then used to determine the constants k. These values were then used in the last equation to determine f (table 2). Based on gross caloric values of 3.40, 4.96 and 5.95 kcal/gram for acetic, propionic and butyric acid, respectively (16), calculations indicated that the VFA disappearing from the cecum were equivalent to 2.83 kcal per day.

The last meal consumed by the rats averaged 16.6 g which is equivalent to 60.1 kcal (the diet contains 3.62 kcal/gram). The percentage of dietary energy converted to VFA energy is thus (2.83 ÷ 60.1) × 100 or 4.7%. According to Kleiber, the energy requirement for maintenance is 70 × body weight in kilograms raised to a power of 0.75 (17). Applying this formula to the average body weight of the rats at the time of the last meal, the maintenance energy requirement is thus $70 \times 0.337^{0.75}$ or 31.01 kcal. VFA energy from the ce[illegible] can therefore supply (2.83 ÷ 31.01) ×

[5] Autoclips, purchased from Clay-Adams, Inc., New York.

[6] Scintillation detector counter was a Unilux model of Nuclear-Chicago Corp., Chicago, Ill.

[7] POPOP represents p-bis(2-(5-phenyloxazolyl))-benzene.

[8] PPO represents 2,5-diphenyloxazole. Both PPO and POPOP were purchased from Nuclear Chicago Corp., Chicago, Ill.

TABLE 2

$T_{1/2}$, Q_0 and k values used for calculating f, the quantity of VFA disappearing every 24 hours from the rat's cecum

Parameter	Acetic	Propionic	Butyric
$T_{1/2}$, hr	12.1	12.0	7.2
Q_0, mg	31.83	6.92	17.3
k, constant	0.05727	0.05775	0.09625
f, mg/24 hr	415.2	89.9	162.0

or 9.4% of the energy required for maintenance.

That VFA were absorbed and metabolized was shown by the recovery of labeled CO_2 in the air expired following introduction of radioactive VFA into the cecum. Within 2.5 hours 55% of the radioactivity injected into the cecum as acetate appeared in the breath. When propionic and butyric acids were injected the proportions were 46 and 38%, respectively (fig. 4). These values also indicate that the $T_{1/2}$ of VFA is closer to 2.5 hours, especially for acetic acid, than to the values shown in table 2. An inherent bias for the $T_{1/2}$ could not be avoided in the first method because it assumed that no production of VFA occurred after time zero. This assumption automatically underestimated the disappearance rates of these acids. The production of acids prevented the curves (figs. 1, 2, and 3) from declining as fast as they should. Other sources of VFA that could have contributed to the cecal pool and further prevented the decline of the curve were blood, cecal tissues and digesta from other alimentary compartments, especially the lower segment of the small intestine. Contributions from blood and cecal tissue are perhaps quantitatively unimportant because only minute quantities of radioactive VFA were detected in the cecum of rats subsequent to the intravenous injection of radioactive acetate. Not more than 0.3% of the injected acetate radioactivity could be detected in the cecal digesta 1 hour after injection.[9] However, the contribution from the passage of digesta from the small intestine could be appreciable when based on the quantity of VFA found in this segment of the tract.[10]

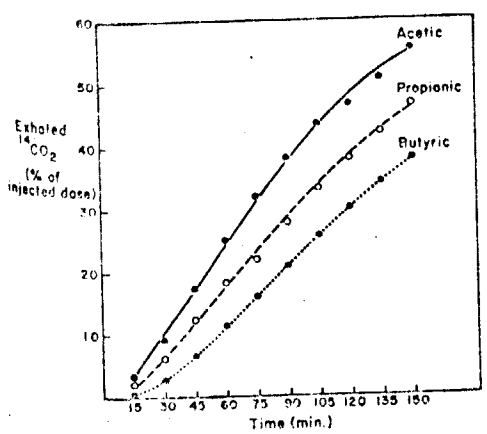

Fig. 4 Appearance of respiratory $^{14}CO_2$ after radioactive VFA were introduced in the cecum of rats.

Even though acetic acid $^{14}CO_2$ appeared in the breath at a faster rate, this does not necessarily indicate that this acid was absorbed and/or metabolized faster than the other two VFA. However, the appearance of radioactivity in the breath strongly suggests that cecal VFA can be used as energy sources. These results are in agreement with those found by Buchanan et al. (10) when radioactive CO_2 appeared in the breath of rats fed radioactive VFA.

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A Continuous Processing Method for Coloring Frankfurters

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SUMMARY

A method of applying an FDA-approved color dye to frankfurters prior to smoking and cooking was developed using a mechanical spraying device. There was no color penetration of the dye. This was due to its use in an acid medium which served to coagulate the protein surface of the raw frankfurter. No differences were noted in color scores due to the use of acetic acid (5%), citric acid (5%) or liquid smoke (20%). Highly significant differences ($P \leq .01$) due to the concentration of dye and length of time the frankfurters were exposed to the spray were recorded. No adverse effects on flavor of the frankfurters by the various treatments were reported by panel members. The use of acids had no effect on bacterial numbers of frankfurters stored for 14 days at 4°C. Panel members selected frankfurters treated with liquid smoke and 3% dye for a period of 30 sec as having the most desirable surface color.

INTRODUCTION

The acceptance of meat depends upon its general appearance with color being most important. Palmer et al. (1962) stated that color of bologna was one of the main factors for consumer acceptance. The color of frankfurters is especially important since the consumer has little else to evaluate before opening the package. It has long been a practice of some meat processors to add artificial color to enhance the natural meat color of frankfurters by processing them in colored artificial casings. Since the removal of FD&C Red #4 by the Food and Drug Administration from its list of approved colors, the industry has been searching for an acceptable replacement. None has been found that will remain stable (i.e., will not oxidize) when stored for a length of time in artificial casings.

Sair (1965) reported that the addition of acid to a sausage during emulsification improved the natural color development of the meat emulsion. Chipley et al. (1968) found that immersing frankfurters in various concentrations of phosphoric, acetic and citric acid and liquid smoke improved natural meat color and removal of artificial casings, but slightly lowered flavor scores as determined by a taste panel. Saffle et al. (1964 a,b) reported other factors that affect the removal of casings of "peeling performance" of frankfurters.

This study was conducted to determine the feasibility of applying an FDA-approved food coloring dye, Orange B-Stange, (the disodium salt of 1-(4-sulfophenyl)-3-ethyl carboxy-4(-4 sulfon-aphtylazo)-5-hydroxy pyrazol) by spraying the raw product prior to cooking. Spraying was introduced because of its adaptability to continuous processing techniques. The effects of concentration and time exposed to the dye, acid, and liquid smoke on surface color intensity, flavor, peeling performance, color penetration and color fading of the frankfurter were determined.

MATERIALS AND METHODS

A standard frankfurter formulation of beef and pork was used to evaluate the effects of added color. Fat determinations were made on all batches prior to emulsification in order to standardize the formulation. All batches analyzed 20% ± 0.6% fat. The meat and ingredients were emulsified and processed in the usual manner prior to stuffing into 24 mm NoJax artificial casings. Following stuffing, the frankfurters were subjected to the treatments listed in Table 1. After the addition of artificial color, the frankfurters were processed in an Alkar smokehouse until the internal temperature of the frankfurters reached 68.3° (approx. 45 min schedule), then showered and placed in a 4°C cooler overnight.

Table 1. Average panel scores for peeling ease of frankfurters.

Treatment	Control	Acetic acid	Citric acid	Liquid smoke
1	7.22	7.46	6.80	7.03
2		7.06	6.60	6.76
3		7.43	6.70	6.93
4		7.36	6.96	6.76
5		7.23	6.50	6.83
6		6.43	6.60	6.60
Average		7.16	6.69	6.82

Treatment 1—no dye added, sprayed with acid for 30 sec.
2—no dye added, sprayed with acid for 90 sec.
3—0.1% dye added, sprayed for 30 sec.
4—0.1% dye added sprayed for 90 sec.
5—3.0% dye added, sprayed for 30 sec.
6—3.0% dye added, sprayed for 90 sec.
Control—no acid or dye

The dye was applied in an acid medium for two reasons: 1) it is more stable in an acid pH and 2) acid cause a slight denaturation of surface protein on the frankfurter eliminating penetration of the dye into the raw product.

The frankfurters were sprayed by passing through a cabinet in which a fine, continuous spray was dispensed through six nozzles, simulating as closely as possible actual conditions in a continuous frankfurter processing line.

After cooling overnight, the frankfurters were evaluated by nine panelists for peeling characteristics, flavor, surface color intensity and color penetration. Peeling characteristics were evaluated as described by Saffle et al. (1964 b). A nine-point hedonic scale was used to evaluate flavor (extremely flavorful = 9, off-flavor = 1) and a 9 point scale (extremely dark = 9, extremely light = 1) used for evaluating surface color intensity. Color penetration was observed by cutting across the frankfurter and examining the cut surface.

Two additional evaluations were made on frankfurters that were held up to 21 days in storage (4°C) to estimate shelf life. All frankfurters were wrapped in Saran film overwrap prior to storage. Total bacterial counts were made on frankfurters from all treatments after 21 days in storage at 4°C. Five swabs taken from 1 cm areas of each of two frankfurters were plated onto tryptone glucose extract (TGE) agar for total plate counts.

The second evaluation on sprayed frankfurters was measurement of conversion or oxidation of the dye over a 14-day period following processing. The procedure used was an adaptation of the method of Dolinsky et al. (1962) for extracting organic colorants from foods. Twenty-five g of sprayed frankfurters were blended with 75 ml of 5% acetic acid and 50 ml resin-in-hexane solution for 5 min, then centrifuged at 5000 × g for 10 min. Aliquots of the supernatant were removed and the absorption at 467 mμ measured in a recording spectrophotometer (Hatachi 12). Readings were compared with a standard curve developed to measure total amounts of the dye.

Table 2. Total counts of bacteria on frankfurters after storage for 21 days at 4°C (×10³).

Treatment[1]	Control	Acetic acid[2]	Citric acid[2]	Liquid smoke[2]
	13.14	2.04	13.86	390.0
		2.84	13.51	321.6
		11.10	25.33	201.5
		11.90	16.03	333.8
		9.65	13.00	800.3
		9.11	5.43	511.6
Average		7.77	14.53	426.5

[1] See Table 1.
[2] Each value represents average of 10 samples.

Table 3. Change in optical density representing oxidation of dye on frankfurters after 14 days storage.

	Change in optical density[2]		
Treatment[1]	Acetic acid	Citric acid	Liquid smoke
3	.36	.35	.52
4	.35	.30	.53
5	.45	.28	.49
6	.40	.42	.57

[1] See Table 1.
[2] Each figure represents the average of 24 samples.

Table 4. Average color scores for dye levels and times of exposure to spray for frankfurters treated with liquid smoke.

Exposure times	Dye levels (%) 0	0.1	3.0	Ave
30 sec.	2.26	3.00	6.33	3.86
90 sec.	2.56	3.85	6.85	4.42
Ave	2.41	3.43	6.59	

RESULTS AND DISCUSSION

Peeling ease. There were no significant differences in peeling ease of the frankfurters among the various treatments within an acid group (Table 1). However, the average peeling scores for each acid group and control were: control—7.22, citric acid—6.69, liquid smoke—6.82, and acetic acid—7.16, indicating that treatment with citric acid prior to cooking was preferential to the other acid-liquid smoke used. This is in contrast to results reported by Chipley et al. (1968) that liquid smoke gave poorer peeling performance than did treatments with acetic acid. Chipley et al. (1968) also indicated that immersion time of 30 sec resulted in a higher mean score than did 90 sec immersion time. It was observed that all frankfurters had reasonably good peeling characteristics, including control. The effects of peeling ease may have been more dramatic if the control frankfurters had lower peeling scores. From this phase of the study it can be concluded that any of the acid/liquid smoke tested could be expected to improve peeling performance of frankfurters.

Flavor. The spice manufacturer's recommended amount of spices was used in formulation and the overall flavor of the frankfurters was not affected by the acids or dyes at the levels used. Taste panel members preferred the flavor of frankfurters treated with citric acid (mean score 5.24) over those receiving liquid smoke (4.65) or acetic acid (4.41); however, these differences were not statistically significant. In studies conducted previously by Chipley et al. (1968) the amount of spices used was reduced by 75% because it was not known how the acids would affect flavor. After observing the scores of taste panel members, they concluded that reduction of spices resulted in lower flavor scores when compared to commercial frankfurters. From the results obtained in this study, it is evident that the acids used did not affect flavor scores.

Color penetration. The purpose of this investigation was to determine if there was any penetration of the dyes beyond the surface of the skin formation. Coloring of frankfurters usually has been accomplished following cooking when the surface skin has already formed. There was no penetration of the dye beyond the skin surface in any of the treatments tested. The lack of penetration of the dye into the meat can be explained by the ability of the dye to attach itself to the protein surface or skin of the frankfurter. By applying the dye in an acid medium the surface protein is partially denatured, thereby preventing penetration of the dye beyond the surface. When FD&C Red #2 was used, the edge of penetration migrated to the center of the frankfurter, probably due to the smaller size of the dye particles and decreased affinity for attachment to the surface protein.

Bacterial counts. Total bacterial counts are shown in Table 2. There were no inhibitory effects by the acids in any of the treatments used. Total bacterial counts were 10 times greater for liquid smoke-treated frankfurters when compared with acid-treated frankfurters. No apparent reason can be given for the higher number of bacteria when using liquid smoke inasmuch as liquid smoke is very inhibitory towards all microorganisms. Pahner et al. (1962) observed that the color additive in bologna began to mask aroma and flavor after 16 days of storage at 38°F. They also found that bacterial deterioration began as soon as the 12th day. Chipley et al. (1968) stated that total bacteria and yeast counts were not adversely affected by acid or liquid-smoke treatments. There was no mold found on any of the refrigerated packages in the present study.

Color-fading as measured by oxidation of dye. There was a significant change in the surface color of frankfurters stored 14 days at 4°C (Table 3). Frankfurters treated with dye plus acetic or citric acid showed less oxidation of dye than did those treated with liquid smoke. This agrees with the findings of Chipley et al. (1968) that color acceptability decreased slowly during a 15-day test period with frankfurters treated with various acids and that fading of color was greater for frankfurters treated with liquid smoke. Pahner et al. (1962) indicated that in light-reduced fading studies, colored bologna retained the desirable color for a longer period of time than did uncolored bologna. Frankfurters colored with artificial coloring retain their desirable color longer than uncolored frankfurters.

Surface color. In comparing the color of frankfurters treated with liquid smoke and various dye levels for 30 or 90 sec, there was a highly significant difference between dye levels (Table 4). The average scores for the various dye levels were 2.41 for the 0% level, 3.43 for the 0.1% level, and 6.59 for the 3% dye level. There was also a highly significant difference in color scores due to length of time the frankfurters were exposed to the dye in showering, 3.86 for those exposed 30 sec and 4.42 for those exposed 90 sec. A highly significant difference was also noted due to variation among panelists, with average panel scores ranging from 2.33 to 5.11. As could be expected, there was also a highly significant interaction between the way individual panel members scored the color of the frankfurters and the concentration of dye used for coloring.

In comparing color scores resulting from treatment with liquid smoke and citric acid using frankfurters from the same batch, there were indications ($P \leq .05$) of a difference due to treatment between the two treatments with means of 4.06 for liquid smoke and 3.89 for citric acid (Table 5). There was no difference between replications and again a highly significant difference in color scores due to dye levels: 0% level—2.51, 0.1% level—3.32 and 3% level—6.10. A highly significant difference was noted due to exposure times or spraying times with average scores of 3.72 for 30 sec and 4.23 for

Table 5. Average color scores for dye levels and times of exposure to spray for frankfurters treated with liquid smoke and citric acid.

Exposure times	Citric acid (5%) 0	0.1	3.0%	Liquid smoke (20%) 0	0.1	3.0%	Ave
30 sec	2.39	3.06	5.61	2.22	2.78	6.27	3.72
90 sec.	3.06	3.61	5.61	2.39	3.83	6.88	4.23
Ave	(Citric acid)		3.89	(liquid smoke)		4.06	

Table 6. Average color scores for dye levels and times of exposure to spray for frankfurters from two different batches treated with citric acid.

Exposure times	Batch 1 0	0.1	3.0%	Batch 2 0	0.1	3.0%	Ave
30 sec.	2.44	3.78	6.44	2.22	3.33	6.00	4.04
90 sec.	2.56	4.22	7.11	2.56	3.44	5.89	4.30
Ave	(Batch 1) 4.43			(Batch 2) 3.91			

Table 7. Average color scores for dye levels and times of exposure to spray for separate batches of frankfurters treated with acetic acid.

Exposure times	Batch 1 0	0.1	3.0%	Batch 2 0	0.1	3.0%	Batch 3 0	0.1	3.0%	Ave
30 sec	3.22	5.00	7.67	2.44	4.00	7.33	2.11	3.56	4.89	4.40
90 sec	3.56	5.11	7.56	2.67	4.89	7.00	2.44	3.89	6.11	4.80
Ave	(Batch 1) 5.33			(Batch 3) 4.63			(Batch 3) 3.83			

90 sec. A highly significant interaction was also present between the acid/liquid smoke and the dye levels used. The taste panel members scored as their most acceptable choice of color those frankfurters treated with liquid smoke and 3% dye for 30 sec.

To make comparisons between batches made on different days, two separate batches of frankfurters were prepared 2 weeks apart and treated with citric acid and dye together (Table 6). There was a highly significant difference between color scores due to batches being made on different days (batch 1—4.43 ave panel score, batch 2—3.91 ave panel score). This was probably due to the variations that occur in day-to-day scoring by individual panel members. Again, highly significant differences were noted due to dye levels (0%—2.44, 0.1%—3.69, 3%—6.36) and exposure time (30 sec—4.04, 90 sec—4.30). A highly significant interaction occurred between batches and dye levels used.

To further investigate differences in scoring of color dye with different batches made on different days, three separate batches were manufactured and treated with acetic acid and dye together. Again, highly significant differences were recorded among scores from different batches (batch 1—5.33, batch 2—4.63, batch 3—3.83) (Table 7). As in previous comparisons, highly significant differences occurred among dye levels (0%—2.72, 0.1%—4.41, 3%—6.67) and between times of exposure to the dye (30 sec—4.40, 90 sec—4.80). A highly significant interaction existed between batches and dyes.

It can be concluded that there were no differences in color of frankfurters due to the acid/liquid smoke treatments but color intensity was a function of the concentration of dye and length of time the frankfurter was exposed to the dye. Variation in color scores that exists among batches of sausage made on different days emphasizes the fact that inherent variations do exist in different batches of any fabricated foods and this should be taken into account when designing experiments of this type. Variation also existed in the way panel members scored the same frankfurters and also in the way they scored similarly treated frankfurters on different days. A highly significant interaction existed between scores of frankfurters made on different days and the individual scoring of different panel members indicating the need for maximum control of these variables when conducting experiments involving panel members. This is emphasized by the fact that although there were no significant differences among the color scores of control (untreated) groups, there was a highly significant difference among panel member's scoring of these controls. Panel members selected frankfurters treated with liquid smoke and 3% dye for 30 sec as having the most desirable color.

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NOTICE: Page Charges for Publication of Research Papers

☐ AT ITS *March 1969 meeting, the Executive Committee of IFT voted to increase the page charge for research articles published in* Food Technology *or in the* Journal of Food Science. *The page charge of $50 per printed page will be effective for research manuscripts received AFTER April 1, 1969. The page charge* shall not *constitute a bar to acceptance of research manuscripts because the author is unable to pay the charge.*

Polyunsaturated Fatty Acids of Skin; Identification and ^{14}C-Acetate Incorporation

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ABSTRACT

Following exposure of the tissue to ^{14}C-acetate, polyunsaturated fatty acids were separated from the total fatty acids of newborn human preputial and mouse skin using argentative thin layer and gas liquid chromatographic procedures. They were characterized by conventional methods including oxidative cleavage and gas liquid chromatography of fission fragments. Members of the linoleic and linolenic acid classes were present in labeled form including the parent acids. Linoleic and arachidonic acids predominated, all others occurring in relatively small amounts. Patterns for the two types of skin were similar in distribution of both mass and radioactivity of individual acids.

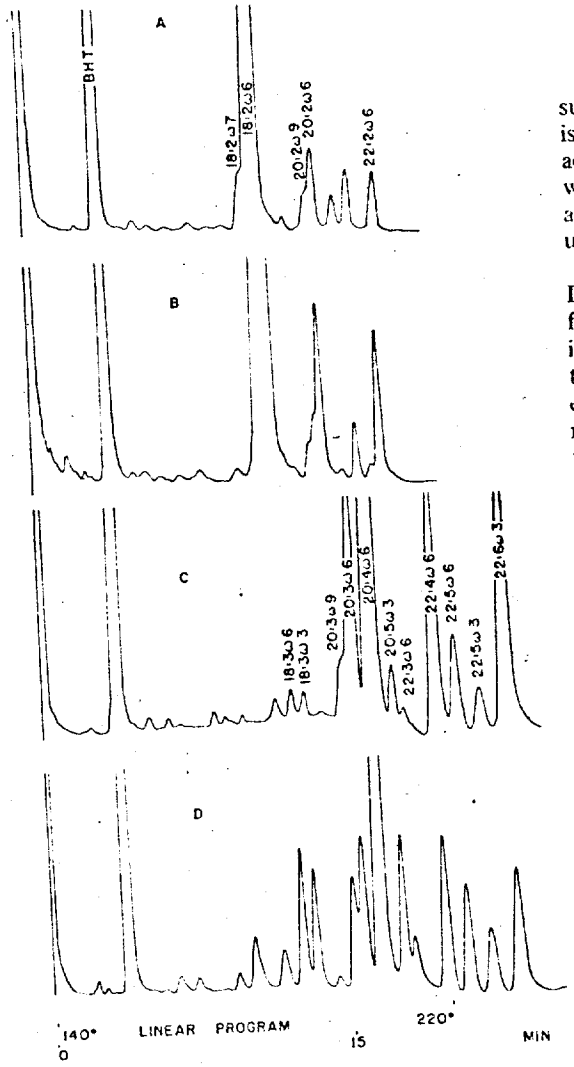

FIG. 1. Gas liquid chromatography traces of dienes (A and B) and polyenes (C and D) from preputial and mouse skin, respectively.

Apart from diene fatty acids of human skin surface lipids (sebum, Ref. 1), little information is available about the polyunsaturated fatty acids (PUFA) of skin. Recently 18:2 and 20:4 were identified in lipids of human preputial and abdominal skin (2), but other PUFA remained undetected.

This report describes the characterization of PUFA in skin and their radioactivity levels following exposure of the tissue to ^{14}C-acetate in vitro. In an earlier communication, study of the incorporation of acetate into octadecadienoic acids in the lipids of epidermal cells released from human skin by trypsin revealed that labeling was associated unexpectedly almost exclusively with the ω6 isomer, linoleic acid (3). As trypsinization might have modified the cells' enzymatic potential (4), labeling of 18:2 was then studied with intact skin, and the results are reported here.

The present study utilized newborn human and mouse skin because desaturation enzymes show maximal activity in very young tissues (5). However, among the ^{14}C- labeled fatty acids of skin from young mice, Brooks et al. found that linoleic acid remained unlabeled (6). It was necessary to determine whether or not this represented a difference in PUFA metabolism between mouse and human skin.

MATERIALS AND METHODS

Skin

Preputial skin speciments were from the nursery; connective tissue was trimmed off. Newborn mice, strain BALB/c, 1-2 days old were sacrificed, and their skin removed. All tissue was rinsed with saline, blotted dry and weighed.

TABLE I

Occurrence and Radioactivity Distribution among PUFA

Fatty acid or class	Preputial skin: Occurrence, % of total PUFA[a]	Preputial skin: Radioactivity, % of total of PUFA[a]	Preputial skin: Specific activity cpm/μg	Newborn mouse skin: Occurrence, % of total PUFA[a]	Newborn mouse skin: Radioactivity, % of total of PUFA[a]	Newborn mouse skin: Specific activity, cpm/μg
Saturates	—[b]	57750[c]		—	19593[c]	
Monoenes	—	6875[c]		—	3211[c]	
Dienes	43.4[d]	3592[c]	83	415.8[d]	1530[c]	3.7
Polyenes	76.3[d]	2899[c]	38	305.8[d]	2479[c]	8.1
18:2ω6, 18:2ω7	31.7	40.2	60	50.7	22.5	2.3
20:2ω6, 20:2ω9	1.5	13.5	430	3.2	10.1	16.3
22:2ω6	0.5	1.5	148	1.9	1.2	3.2
18:3ω6	0.6	3.3	268	2.0	7.7	20.1
18:3ω3	0.3	1.1	124	2.1	4.1	10.6
20:3ω9	0.7	—	—	2.4	—	—
20:3ω6	4.4	21.1	227	2.8	15.9	30.6
20:4ω6	38.4	2.6	3	20.5	2.1	0.5
20:5ω3	1.1	0.4	14	2.2	0.3	0.7
22:3ω6	0.4	0.6	60	1.1	1.1	5.2
22:4ω6	5.3	10.7	96	2.6	11.6	25.0
22:5ω6	1.8	2.2	58	1.9	2.5	6.8
22:5ω3	0.9	2.1	111	0.9	2.7	14.0
22:6ω3	5.1	1.2	11	1.6	4.5	14.7
Others	6.6	3.3		4.8	2.9	

[a]Figures are per cent of total amounts for PUFA (dienes + polyenes) except where specified otherwise.
[b]Mass of saturated and monoene classes not determined.
[c]In cpm/100 mg skin.
[d]In μg/100 mg skin.

Radioactive Materials

Sodium 1-^{14}C-acetate, 54 mCi/mM, and linoleic acid, 10 mCi/mM, methylated with diazomethane, were used.

Labeling

Each preputial or mouse skin was minced with scissors and incubated at 37 C in air in a vial containing 2 ml Pucks Saline G, pH 7.4, with 5 μCi of sodium 1-^{14}C-acetate and antibiotics (7) for 4 hr, using a shaking incubator.

Isolation of Methyl Esters

After labeling, tissue was homogenized in $CHCl_3/CH_3OH$ 2:1. The homogenate was filtered through fritted glass and the solvents removed in a rotary evaporator at 50 C. Ten preputial and 15 mouse skins were labeled and extracted in this manner, and the residues after solvent removal were pooled to obtain two large lipid samples. Methylation and purification of methyl esters by thin layer chromatography (TLC) on silica gel has been described before (7), except that benzene was used here to develop the plates in one step. Purified esters were resolved by argentative TLC. Plates were developed with 0.75% CH_3OH in $CHCl_3$ (8) and ester bands visualized with 0.2% ethanolic 2,7-dichlorofluorescein. This process separated saturated, monoene and diene bands from a fourth band which consisted of the remaining, poorly resolved polyenes. These four bands were scraped from plates, extracted with CH_2Cl_2/CH_3OH 4:1, silica gel filtered off, the filtrate evaporated and the residue extracted with pentane. Bands from each plate were pooled. The fourth fractions (pooled) were reapplied to a single plate, developed with hexane-ethyl ether 4:1 and resolved into tri-, tetra-, penta- and hexaene fractions which were recovered as before. Samples of pure methyl esters of known unsaturation were run alongside.

Each sample of PUFA of defined unsaturation was resolved into its component methyl esters by small scale preparative gas liquid chromatography (GLC) as described before (7). Samples of 1 mg or less were injected. The column was operated either at 182 C or linearly programed from 140-220 C. Emerging peaks were collected in glass tubes as before (7) and recovered by rinsing the tubes with hexane. An aliquot of each hexane solution was evaporated and redissolved in scintillation fluid for radio-

activity assay.

Saturated and monoene fractions were not subjected to GLC, but extracted from the $AgNO_3$/silica gel, dissolved in scintillation fluid and counted.

Hydrogenation

Aliquot samples of the hexane solutions of individual methyl esters or classes of esters were evaporated, and the residue was hydrogenated as before (9). Products were analyzed on the same GLC column as above.

Double Bond Location

Individual PUFA were oxidized by $NaIO_4$/$KMnO_4$ as before (3), or by reductive ozonolysis (10), or by both methods. Solutions containing oxidation fragments (after diazomethane methylation of carboxyl groups when applicable) were concentrated to 25 μliters and injected into a GLC column similar to the one used above (10% EGSS-X on Gas Chrom P, 100/120 mesh). Injections were made at 130 C column temperature (injector 230 C, detector 220 C, helium flow 38 ml/min) which was programed to 200 C at 4 C/min. Peaks were collected in glass U-tubes, 3 mm ID cooled in acetone-CO_2. Finally each tube was rinsed with scintillation fluid into vials for counting.

Reagents

Solvents were reagent grade, distilled before use and with BHT added (0.001%). Fatty acid samples were kept under nitrogen as much as possible. Ozone was obtained from a corona generator with oxygen flow of 2-3 ft^3/hr. Scintillation fluid has been described (7), and counting was performed in a Beckmann LS-100 instrument, with 90% efficiency. Samples of PUFA were obtained from Schwarz-Mann, Sigma and Nu Chek Prep, Elysian, Minn. 56028.

RESULTS AND DISCUSSION

In skin lipids, saturated and monoenoic fatty acids predominate, and the bulk of radioactivity incorporated from ^{14}C-acetate by skin is associated with these fractions (2). Except for 18:2 and 20:4, PUFA are present in small amounts, and therefore tend to be obscured in GLC analysis of a total fatty acid sample by more abundant and highly labeled components.

In this study, resolution of total samples into groups according to degree of unsaturation was accomplished by small scale preparative $AgNO_3$/TLC. Development of these TLC plates with $CHCl_3$/CH_3OH (8) produced satisfactory separation of saturates, mono- and dienes from the remaining polyenes. Resolution of the complete PUFA mixture (dienes plus polyenes) by preparative GLC was not satisfactory due mainly to the obscuring of 18:3 peaks by the very large 18:2 peak. However separate GLC treatment of dienes and remaining polyenes provided good resolution of most peaks (Fig. 1). For complete characterization of individual peaks the polyene fraction was separated on a second $AgNO_3$/TLC plate. Morris has described a two-stage separation of saturated and unsaturated (mono- through hexaenes) methyl esters on the same plate (11), but in our hands his method proved less satisfactory.

The efficiency of condensation of methyl 1-^{14}C-linoleate in 3 mm glass tubing (95%) was similar to that of methyl stearate (7) over a 5-200 μg range. Efficiency was lowered by use of wider tubes or higher flow rates, e.g., when use of wider columns was attempted.

Individual PUFA methyl esters were subjected to oxidative degradation using $NaIO_4$/$KMnO_4$, and in some cases by ozonolytic fission with reductive workup (10) to confirm results obtained with the former. In previous studies on monoenes the use of mixed GLC phases (7% silicone DC-560 + 1% polyester EGSP-Z) permitted the resolution of mono- and dicarboxylic methyl esters (3). However oxidation of PUFA produces monocarboxylic (or aldehydic) fragments of only short chain length. Thus the difunctional (dicarboxylic esters or aldehyde-esters) fragments alone were used in structure determination and were conveniently separated on an EGSS-X column, a portion of each being collected as described. The efficiency of collection of each and the loss incurred in evaporation of pentane solutions to volumes suitable for injection were studied with a mixture of ca. 100 μg of dicarboxylic esters from C_5 (glutarate) through C_{12}. From the mass traces before and after evaporative and collection procedures it was possible to estimate the overall loss of individual members, such as C_5 (74%), C_6 (43%) and C_7 (25%). Longer chain compounds were recovered almost quantitatively.

The extensive information available on the separation of PUFA on polyester columns was used to predict the identity of many PUFA in this study (12,13). Further confirmation was obtained by conventional methods including information from patterns of degradation fragments.

The GLC patterns of PUFA from preputial and mouse skin were quite similar (Fig. 1) and resembled patterns recorded for other mammalian tissues (13). This similarity was somewhat surprising in view of the ability of mouse skin alone to synthesize monounsaturated fatty acids with up to 35 carbon atoms (9). However

more highly unsaturated chains of this length have not been demonstrated. PUFA belonging to the ω3, ω6 and ω9 series were observed.

Data on the occurrence and label distribution among component PUFA are given in Table I. Hydrogenation showed only traces of odd-numbered or branched chains present. On the basis of cpm per unit weight of tissue, preputial skin incorporated more label into its fatty acids, especially saturates, than did mouse skin. In terms of μg per unit weight of tissue, however, mouse skin far exceeded preputial skin in amounts of PUFA present. (Determination of mass of saturates and monoenes were not performed in this study; however, from TLC patterns, preputial skin was judged to contain about the same relative amounts of saturates, monoenes and PUFA as mouse skin.)

For both types of skin, oxidation showed that ca. 92% of the 18:2 peak was linoleic acid (18:2ω6), with ca. 95% of its radioactivity associated with the derived C_9 fragment (azaleic acid) and the remainder with a C_8 fragment, which was probably an oxidation byproduct. This confirmed patterns observed earlier with epidermal cells (3). Schmidt degradation of hydrogenated 18:2 (as free acid) revealed that radioactivity was located in the carboxyl carbon. In the absence of evidence to the contrary, carboxyl labeling of 18:2ω6 (and 18:3ω3) is usually attributed to exchange with ^{14}C-acetate (14). A possible C_{16} precursor of linoleic acid (16:2ω6) was not identified in this study, although its existence is a subject of continuing research.

Linoleic and arachidonic (20:4ω6) acids formed ca. 70% of skin PUFA. Of the total PUFA radioactivity, linoleic acid carried the largest share (40-50%), followed by 20:3ω6 (15-21%), 20:2ω6 (10-13%) and 22:4ω6 (10-11%). Obviously the ω6 pathway of PUFA formation predominated, although conversion of 20:3ω6 to arachidonic acid (lightly labeled) was not very significant.

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