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Acute antilipolytic effects of ethyl alcohol and acetate in man

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Ethyl alcohol has been shown to acutely lower plasma free fatty acid (FFA) levels in man. These studies were undertaken to further define the mechanism of this effect and the clinical situations in which the effect is obtained. In normal subjects, infusion of ethyl alcohol at a rate of 5.12 mmoles per minute resulted in an acute fall of plasma FFA and glycerol levels without changes in plasma glucose, insulin, or growth hormone. Alcohol similarly lowered FFA after prolonged fasts, in decompensated diabetes, and during epinephrine infusion. Acetate, a major metabolic product of alcohol oxidation, was infused at 0.155 to 5.12 mmoles per minute. In normal subjects acetate also produced a prompt fall in FFA and glycerol as well as total ketone levels. This occurred independently of the alkalinizing effects of acetate. Plasma glucose, insulin, and growth hormone and blood lactate levels were unaffected. Acetate lowered FFA in subjects with thyrotoxicosis, during epinephrine infusion, and in the presence of beta adrenergic blockade. These studies indicate that alcohol and acetate acutely depress lipolysis in man without producing alterations in measured neurohormonal factors, and they suggest that the effects of alcohol may be mediated by its metabolite, acetate.

Circulating levels of free fatty acids reflect the equilibrium between hydrolysis and re-esterification of triglycerides in adipose tissue, as well as the balance between peripheral utilization and adipose tissue release of these substances. A number of metabolic and pharmacologic agents such as glucose, insulin, and nicotinic acid acutely lower the plasma levels of both fatty acids and glycerol by directly or indirectly inhibiting lipolysis.[1] Similarly, physiological factors such as exercise induce a reduction in free fatty acid levels by

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accelerating the uptake of these substances by peripheral tissues? The observation that the administration of ethyl alcohol in man causes a rapid and precipitous fall in fatty acid levels[1] has been the stimulus for studies directed to (1) defining the mechanism and site of this effect.

Although it has been suggested that alcohol[2] interferes with the process of triglyceride hydrolysis,[3-6] it has not been ascertained whether alcohol acts directly on adipose tissue or through an intermediate neurohormonal mediator. Moreover, since alcohol is readily oxidized to acetaldehyde and acetate,[7] the question arises whether the interference with lipolysis reflects an effect of alcohol *per se* or one of its by-products. This communication describes studies undertaken to define (1) the circumstances under which alcohol inhibits lipolytic activity, (2) whether similar effects can be obtained with acetate, a major product of alcohol oxidation, and (3) whether these effects are reproducible in other species.

Materials and methods

All studies were performed on the metabolic ward of the Thorndike Clinical Research Center. Subjects were placed at complete bedrest in an air-conditioned room for one-half to one hour prior to the initiation of study. Unless otherwise specified, studies were conducted after an overnight fast. Healthy, normal weight volunteers were recruited from college and medical students and paramedical personnel. One obese subject, hospitalized for weight reduction, participated in the studies after separate fasts of 3, 11, and 23 days. Subjects with hyperthyroidism were recruited from the Endocrine Clinics of the Boston City and St. Elizabeth's Hospitals. Clinical diagnosis was confirmed by measurement of serum protein bound iodine and 24 hour I^{131} thyroidal uptake. Patients with juvenile type diabetes were hospitalized and placed on regular insulin for control as described earlier.[8] Doses of insulin were withheld for periods of 8 to 16 hours to induce mild decompensation and assure elevation of fatty acid and ketone levels. All individuals were informed of the purpose and nature of the study.

Ethyl alcohol was diluted in sterile saline to make a 2.56M solution. To assure sterility of such solutions, repeated bacteriological cultures were obtained. Sodium acetate solutions ranging in concentration from 0.31M to 2.56M were prepared in distilled water and autoclaved. Alcohol solutions were infused intravenously for one hour at the rate of 2.0 ml. per minute (i.e. 236 mg. or 5.12 mM. per minute), with regulation by a Sigma motor pump. Solutions of sodium acetate were delivered at rates of 0.5 ml. to 2.0 ml. per minute for one hour, corresponding to 0.155 to 5.12 mM. per minute. When given orally, a total of 128 mmole of either sodium acetate or sodium bicarbonate in 50 ml. of water was ingested over a 2 minute period. Solutions of epinephrine[4] in saline were made to permit infusion of 6.0 μg of epinephrine per minute. Propranolol, a specific beta-adrenergic blockade agent, was injected intravenously over a 1 to 2 minute period in doses of 0.15 mg. per kilogram to assess the antilipolytic effect of acetate in the presence of beta-adrenergic blockade. Since this amount of propranolol generally lowered free fatty acids (FFA) to less than 350 μEq per liter when given after overnight fast, studies with propranolol were conducted after 36 hour fasts. In the control study, subjects received propranolol

[1] The terms alcohol and ethyl alcohol are used synonymously throughout the paper.
[2] Adrenalin Chloride, Parke, Davis & Company, Detroit, Mich.
[3] Inderal, supplied through the courtesy of Dr. A. Sonnenschwards, Ayerst Laboratories, Inc., New York, N. Y.
[4] This dose completely blocks the lipolytic effect of epinephrine infused at 6.0 μg per minute for at least one hour. During epinephrine infusion at this rate in 5 subjects, FFA values at 1, 5, 10, 20, 30, 40, 50, 60 minutes were 105, 285, 397, 397, and 288 per cent of control, respectively. By contrast, FFA levels during epinephrine infusions preceded by this dose of propranolol were 93, 97, 95, and 105 per cents of control, respectively, at the same time points.

alone; while in the second study, an infusion of sodium acetate (1.2 mM. per minute) was started 30 minutes after the injection of propranolol and continued for one hour. The order of these studies was randomized.

Venous samples were drawn without stasis, placed immediately in heparinized tubes, and then separated in a refrigerated centrifuge at 4° C. Methods utilized for analyzing plasma glucose, FFA, glycerol, insulin, and growth hormone are described in an earlier publication.[9] Whole blood for lactic acid analysis was precipitated immediately in cold 3.4 per cent perchloric acid and the protein-free filtrates stored at -18° C. These filtrates were analyzed enzymatically for lactic acid.[10] Ketones were determined by the method of Bessman,[11] with protein-free filtrates of plasma. All samples were analyzed in duplicate and samples from comparative studies were run simultaneously.

Plasma for determination of free fatty acid composition was extracted with a chloroform:methanol (2:1, v/v) extract. The basal extract was evaporated under nitrogen at 50° C. and the residue redissolved in heptane-diethyl ether (1:1 v/v). The solvent was reduced to 0.5 ml. by evaporation under nitrogen at room temperature and placed on silica gel thin-layer plates. The plates were developed for 50 minutes in a petroleum ether: diethyl ether:glacial acetic acid (82:18:1) system. The free fatty acid spots, identified by exposure to iodine vapors, were scraped into a mixture of ethanol and 25 per cent aqueous KOH, which was heated in a 55° C. water bath for 20 minutes to effect saponification. The solution was acidified with 50 per cent $HClO_4$, extracted with hexane, and evaporated under nitrogen. Methyl esters which were formed by heating the residual dissolved in 5 per cent perchloric acid-methanol at 55° C. for 20 minutes were then extracted with hexane-diethyl ether (1:1 v/v) and evaporated at room temperature under nitrogen. For identification, the methyl esters were redissolved in chloroform, and 3 lambda of this solution was chromatographed on an F and M High Efficiency Gas Chromatograph 402 programmed from 160 degrees to 200 degrees at 5° C. per minute on six column DEGS (10 per cent LAC 728). An internal standard of 25 mg. per cent methyl heptadecanoate in chloroform was used.

Animal studies were performed with male Charles River rats, fasted for 48 hours prior to the study. The rats received intraperitoneal injections of either 0.2 mmole of ethanol or acetate, 1.0 mmole of acetate, or an equimolar amount of saline. Total volume of injection was 1.0 ml. in all cases. Ten minutes after the injection the rats were decapitated and the neck vein blood was collected in heparinized beakers. After separation in a refrigerated centrifuge, plasma was analyzed for glucose and free fatty acids as described.[9]

Results

Neither alcohol nor acetate infusions of one hour's duration were associated with subjective discomfort or alterations in pulse, blood pressure, or temperature in any subject. Previous studies have shown that plasma alcohol levels after one hour of similar infusions are usually less than 50 mg. per cent.[7]

Alcohol studies. The effects of intravenous alcohol on glucose, free fatty acids, glycerol, insulin, and growth hormone in a group of 5 healthy, normal subjects are shown in Fig. 1. Plasma levels of FFA fell to a mean (± S.E.M.) of 51 ± 5 per cent of control ($p < 0.001$) after 15 minutes of infusion, while glycerol declined to a mean of 71 ± 6 per cent of control ($p < 0.01$) during the same time interval. Despite continuing infusion of alcohol, both FFA and glycerol rebounded toward control values. Plasma levels of glucose, insulin, and growth hormone underwent no significant change during the alcohol infusion. When 5 normal subjects were infused with saline under identical conditions FFA rose steadily.

Table I demonstrates the effects of alcohol during states of increased lipolysis

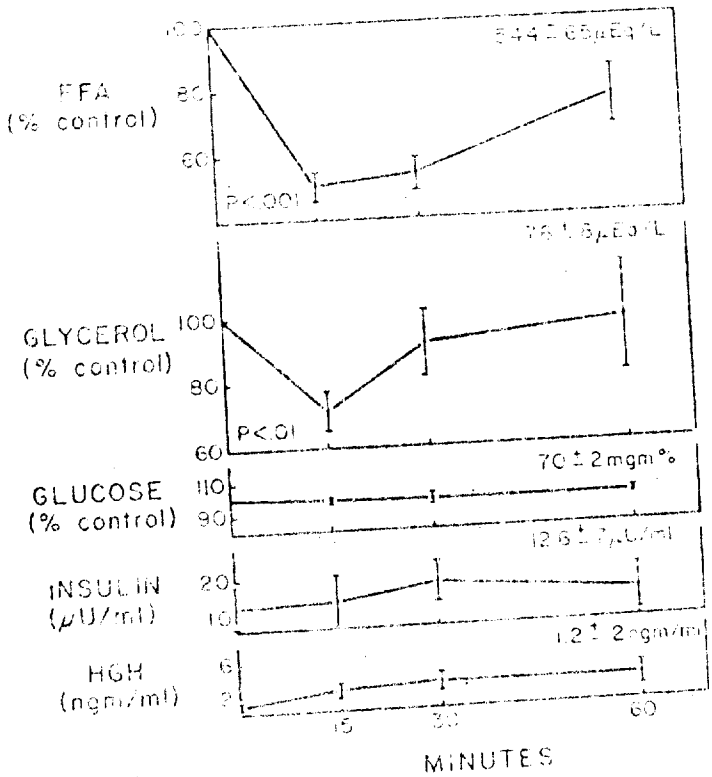

Fig. 1. Effects of intravenous alcohol (236 mg per minute) on free fatty acids (*FFA*), glycerol, glucose, and [illegible] (*HGH*). Each point represents the mean ± S.E.M. of results obtained in 5 healthy volunteers. The values in the upper right of each block are the mean ± S.E.M. of preinfusion values. *P* values in the *FFA* and glycerol blocks refer to the probability at the 15 minute point when compared with the control value.

Table I. Effects of alcohol on plasma FFA in fasting and decompensated diabetes

Subjects	*Control* (μEq/L)	*Percentage FFA* 15 min.	30 min.	60 min.
Fasted normal 5	1,122 ± 154	72 ± [illegible]	61 ± 7	70 ± 10
Ketotic diabetic† 6	1,612 ± 256	65 ± 6	60 ± 5	59 ± 5

*Subjects were fasted for 72 hours prior to the infusion of alcohol.
†Juvenile diabetic patients were controlled on a [illegible] insulin. Ketonuria and [illegible] were [illegible] with [illegible] a single dose of [illegible]

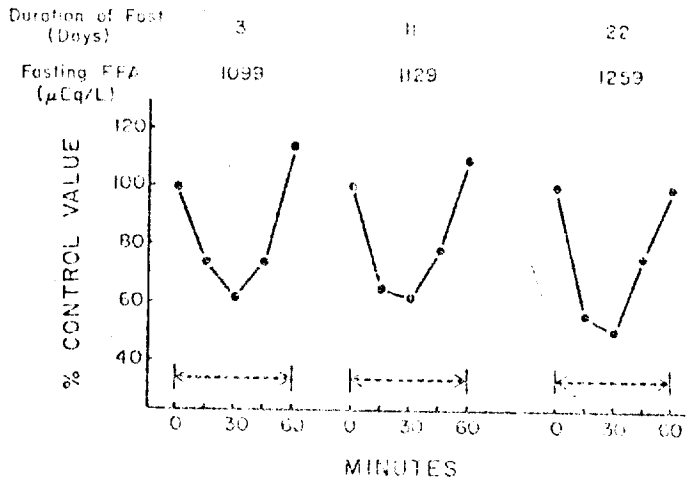

Fig. 2. Effect of alcohol on the *FFA* levels during fasting. The patient, a 54-year-old man, weighing 111.0 kilograms on admission, underwent three separate fasts of 3, 11, and 22 days. Each fast was terminated by an infusion of alcohol (<----->).

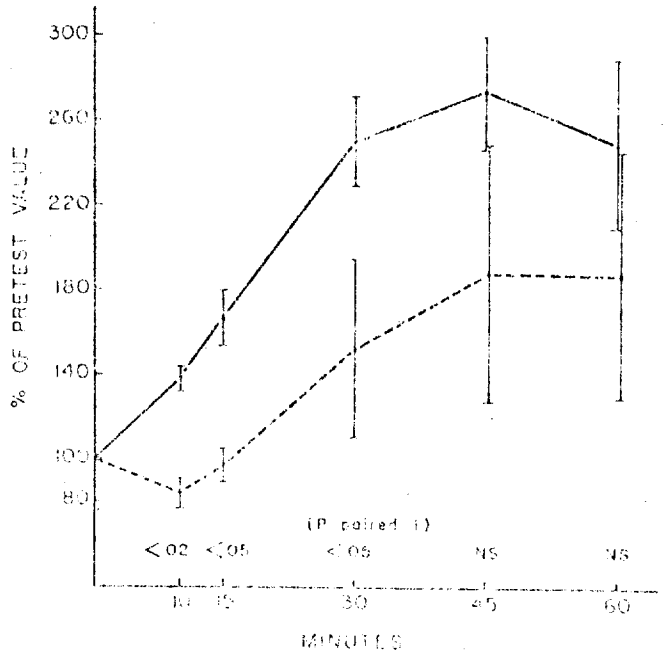

Fig. 3. Inhibition of epinephrine-induced lipolysis by alcohol. Four subjects received infusions of epinephrine (6 μg per minute) as shown by the solid line and similar infusions of epinephrine simultaneously with the standard alcohol infusion as shown by the dashed line. Each point represents the mean ± S.E.M. Control FFA (solid line) 498 ± 72; dashed line 524 ± 68.

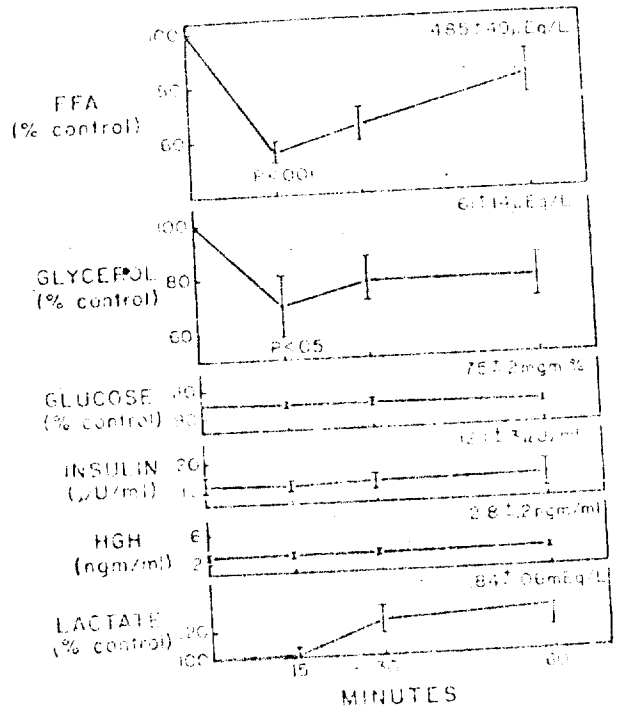

Fig. 2. Effects of sodium acetate infusions on FFA, glycerol, glucose, insulin, HGH, and lactate. Nine normal subjects received infusions varying in amounts from 0.155 to 5.12 mmole per minute. Values in the upper half of each block are the mean ± S.E.M. of pre-infusion values. *P* values refer to the probability after comparison with the control value.

sis. Five volunteers fasted for 72 hours prior to receiving alcohol had a mean preinfusion FFA level of 1,122 ± 151 μEq per liter. Fifteen minutes after initiation of the alcohol infusion, FFA levels had fallen to 72 ± 4 per cent of this value. Similarly, decompensated diabetic patients with a mean FFA level of 1,612 ± 250 μEq per liter had a drop in FFA levels to 65 ± 6 per cent of this value 15 minutes after the start of alcohol administration. During saline infusions, FFA continued to rise in each diabetic patient. In the obese subject, preinfusion FFA values were progressively elevated after separate fasts of 3, 11, and 22 days. On each occasion alcohol infusion resulted in a fall of FFA to 62, 65, and 57 per cent of control, respectively (Fig. 2).

Alcohol significantly inhibited the immediate lipolytic effects of epinephrine (Fig. 3). While this inhibition was evident for 45 minutes, FFA rose thereafter during the alcohol-epinephrine infusions in parallel with those observed during epinephrine infusions.

Acetate studies. Fig. 4 shows the response to intravenous sodium acetate in 9 subjects. Although the rates of infusion varied from 0.155 to 5.12 mmoles per minute, the measured effects were independent of the administered dose in this range. Fifteen minutes after the initiation of the sodium acetate infusion,

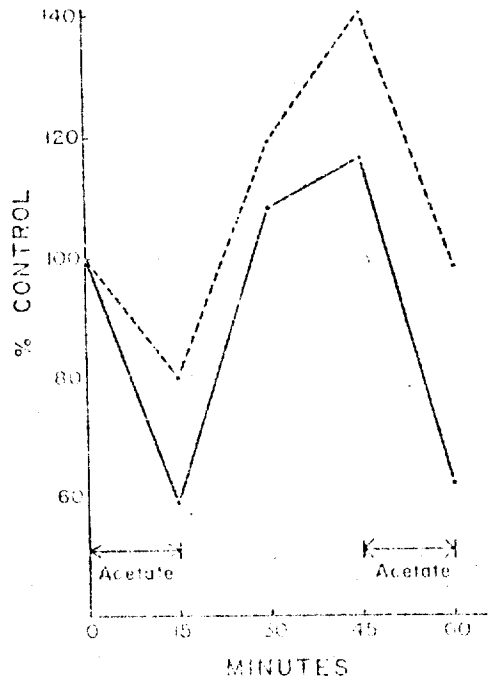

Fig. 5. Effects of repetitive infusions of sodium acetate on FFA levels. Two subjects received sodium acetate infusions of 1.2 mmole per minute for 15 minutes, at which time infusions of saline were substituted for the sodium acetate. Sodium acetate infusions were readministered at the peak of the rebound with a second fall in FFA observed.

FFA levels declined to a mean of 56 ± 4 per cent of control ($p \leq 0.001$), while glycerol fell to 70 ± 11 per cent of control ($p \leq 0.05$). As observed during the administration of alcohol, both FFA and glycerol levels rebounded despite continuing infusion of acetate. However, in 2 subjects when acetate infusion was replaced by saline from 15 to 45 minutes, readministration of acetate at 45 minutes again lowered FFA values (Fig. 5). Plasma glucose, insulin, and growth hormone did not change significantly during acetate infusions (Fig. 4).

Composition of the circulating free fatty acids was not significantly affected by the administration of intravenous sodium acetate and alcohol (Tables II and IIA). When plasma samples taken at the 15 minute point were compared with control samples in regard to the palmitic, palmitoleic, stearic, oleic, and linoleic acid content, the distribution of these acids was unchanged.

Since sodium acetate is an alkalinizing agent, 8 subjects were given oral doses of 128 mmoles of sodium acetate, while 5 subjects received equimolar amounts of sodium bicarbonate. The results of these studies are noted in Table III. Acetate induced its maximum effect 45 minutes after the oral dose, without alteration in venous pH, while FFA rose slightly concomitant with the mild alkalosis induced by bicarbonate.

Table II. Effect of intravenous sodium acetate on specific plasma fatty acids

Subject	FFA (μEq/L.)		Percent total FFA*				
			Palmitic	Palmitoleic	Stearic	Oleic	Linoleic
1	Control	474	34.1	5.2	3.5	26.0	19.0
	15 min.	189	29.6	5.4	2.5	28.0	21.1
2	Control	527	40.1	5.2	2.1	26.8	12.7
	15 min.	150	15.5	2.8	2.2	25.8	11.0
3	Control	511	32.1	4.7	2.1	13.8	11.1
	15 min.	254	14.2	6.6	3.5	26.5	19.0

*Based on the assumption that these 5 fatty acids comprise 75 per cent of the total circulating FFA.

Table IIA. Effect of intravenous alcohol on specific plasma fatty acids

Subject	FFA (μEq/L.)		Percent total FFA*				
			Palmitic	Palmitoleic	Stearic	Oleic	Linoleic
4	Control	484	34.6	5.7	4.2	27.2	13.2
	15 min.	212	29.1	4.3	4.5	28.0	18.3
5	Control	523	40.2	3.8	4.1	26.4	10.6
	15 min.	308	29.0	3.2	3.8	35.8	14.3

Table III. Comparative effects of oral sodium acetate and sodium bicarbonate on pH, FFA, glucose, and insulin

	Control	30 min.	45 min.	60 min.
pH				
Acetate	7.40 ± 0.02	7.41 ± 0.01	7.47 ± 0.02	7.46 ± 0.01
Bicarbonate	7.43 ± 0.01	7.44 ± 0.01	7.47 ± 0.01	7.47 ± 0.01
FFA (μEq/L.)				
Acetate	532 ± 50	399 ± 37	416 ± 43	484 ± 64
Bicarbonate	485 ± 30	529 ± 29	686 ± 29	606 ± 58
Glucose (mg./%)				
Acetate	73.0 ± 2	74.4 ± 2	74.1 ± 2	70.8 ± 2
Bicarbonate	73.0 ± 3	74.8 ± 3	73.4 ± 3	73.8 ± 2
Insulin (μU/ml.)				
Acetate	7.8 ± 2	8.4 ± 2	7.6 ± 2	8.3 ± 3
Bicarbonate	10.6 ± 3	5.4 ± 3	11.6 ± 4	7.0 ± 3

Sodium acetate (5.12 mM. per minute) inhibited the lipolytic action of epinephrine (Fig. 6). In 5 subjects FFA levels increased to 237 ± 4 per cent of preinfusion values after 30 minutes of epinephrine infusion (16.0 μg per minute) and declined to 173 ± 22 per cent of preinfusion values 45 minutes after the addition of acetate. This effect differed significantly ($p < 0.01$) from the progressive FFA rise observed during infusions of epinephrine alone.

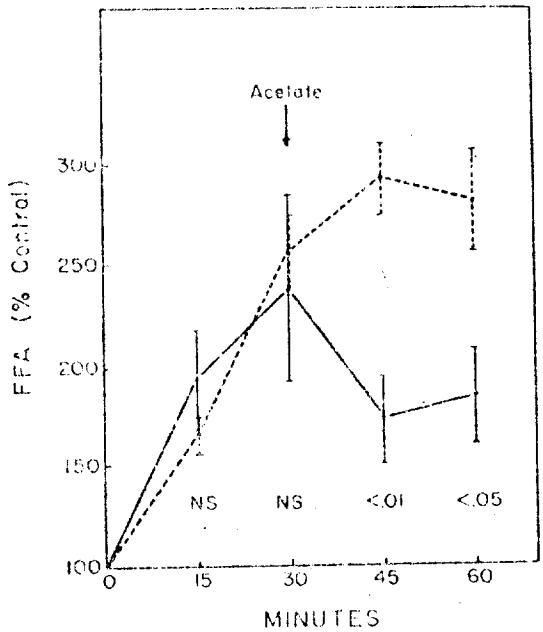

Fig. 6. Effects of sodium acetate on the lipolytic action of epinephrine. Five subjects received a constant infusion of 6 μg epinephrine per minute for one hour (dashed line) and, on another occasion, the same infusion with sodium acetate (2.5 mM) added at the 30 minute point (solid line). Sodium acetate induced a fall in FFA in the face of continuing epinephrine infusion. Control FFA (dashed line) 532 ± 52, (solid line) 458 ± 69.

In 4 subjects infused with 0.155 mM. of sodium acetate per minute, the total plasma ketone levels fell from a mean of 1.68 ± 0.22 mg. per 100 ml. to 1.29 ± 0.21 mg. per 100 ml. at 45 minutes. Plasma lactate levels in this group were 0.84 ± 0.15 mEq. per liter preinfusion and 0.85 ± 0.20 mEq. per liter 45 minutes after the start of the infusion.

Augmented lipolysis likewise was depressed in 3 thyrotoxic subjects who had a mean control FFA level of 1,020 ± 66 μEq per liter. Ingestion of 128 mmoles of sodium acetate effected a significant fall of FFA to 79 ± 5 per cent of control levels after 15 minutes.

Addition of acetate infusion 30 minutes after full-blocking doses of propranolol led to a further fall of FFA, significantly different from control studies, $p < 0.01$ (Fig. 7).

Animal studies. Alcohol (0.5 mmole) significantly lowered plasma FFA in rats within 30 minutes of injection, and did not affect plasma glucose (Table IV).

An equal amount of sodium acetate produced a small decrease in FFA and a rise in glucose. Neither was significant. One millimole of acetate did significantly lower FFA, but not as much as alcohol. Moreover, the FFA decline was accompanied by a significant increase in plasma glucose (Table IV).

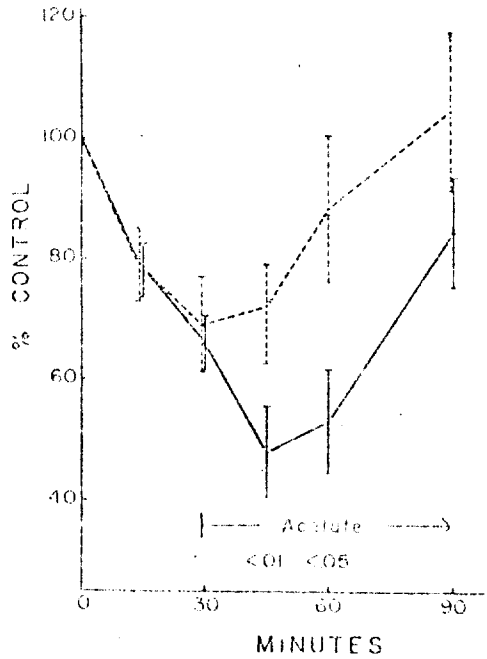

Fig. 7. Effect of sodium acetate on FFA levels following induction of complete beta adrenergic blockade. Complete blockade of beta-adrenergic receptors was accomplished by the intravenous injection of propranolol (0.15 mg. per kilogram) in 3 healthy subjects after a 36 hour fast (dashed line). When similar injections were followed by an infusion of 2.56M sodium acetate at the 30 minute point (solid line), the decline in FFA was significantly augmented. *P* values represent the paired t analysis at the 45 and 60 minute time points.

Table IV. Effects of alcohol and acetate on plasma glucose and FFA

Experiment No.	*No. of rats*	*Injection (mmoles)*	*Plasma FFA (μEq/L.) mean ± S.E.M.*		*Plasma glucose (mg./%) mean ± S.E.M.*	
1	8	Ethanol (0.3)	505 ± 50	$p < 0.01$	99 ± 6	N.S.
	9	Saline (0.3)	806 ± 66		105 ± 5	
2	5	Sodium acetate (0.3)	889 ± 61	N.S.	111 ± 4	N.S.
	5	Saline (0.3)	916 ± 32		106 ± 5	
3	5	Sodium acetate (1.0)	730 ± 46	$p < 0.02$	130 ± 2	$p < 0.01$
	5	Saline (1.0)	913 ± 52		115 ± 3	

Discussion

Both alcohol and acetate acutely lower plasma free fatty acid and glycerol levels in man. This constitutes evidence for an antilipolytic action, especially in the case of alcohol, which has been shown to actually depress glycerol uptake in the splanchnic bed of man.[5] Both agents lower FFA not only in basal states but in a number of pathological, physiological, and pharmacological conditions characterized by augmented lipolysis. In man, alcohol is readily oxidized to acetate, and plasma acetate levels rise promptly after alcohol is administered.[5] Therefore, while the similar effects of these two agents may be coincidental, the data suggest that the antilipolytic action of alcohol may be ascribed to its metabolite, acetate. Nevertheless, it remains indisputable that the administration of acetate acutely depresses lipolysis in man. While our data do not delineate the mechanisms by which acetate acts, several speculations can be made.

In view of the remarkable sensitivity of rat epididymal fat to insulin in vitro,[16] it is conceivable that acetate acts by inducing an increase in plasma insulin. Such a postulate is unlikely since alcohol lowers FFA in decompensated "juvenile" diabetic patients who are unable to increase endogenous insulin secretion, and in several patients infused with alcohol or acetate, plasma insulin actually fell 2 to 3 μU per milliliter in the interval from zero to 45 minutes. Moreover, others[15,17] have demonstrated that alcohol does not elevate plasma insulin levels.

Acetate might act as an alternate metabolic substrate and make increased amounts of glucose available for use by adipose tissue. This could not account for the observed fall in both FFA and glycerol, since glucose alone will not depress lipolysis or glycerol release by adipose tissue.[1] As an alternate substrate, acetate might spare FFA utilization in peripheral tissues, but in the face of unaltered lipolysis, FFA levels would be expected to increase, unless peripheral tissues could "signal" adipose tissue that their needs were satiated. Furthermore, acetate has been shown not to depress utilization of palmitate, a representative fatty acid, in the perfused rat heart.[5]

Goodner[1] has shown that minute amounts of glucose acutely lower FFA in man, and he has attributed this to the dampening of autonomic tone. Since it has been shown that the human central nervous system can utilize certain lipid metabolites, i.e., ketones,[18] it seemed possible that acetate, acting as an alternate substrate at a neural center, might similarly dampen autonomic tone. However, since acetate retains antilipolytic properties in the presence of full beta-adrenergic blockade, such a mechanism would appear to be inoperative in these studies.

Recent work of Björntorp[4] has documented the antilipolytic effect of beta-hydroxybutyrate in the pancreatectomized dog. Such an effect has yet to be documented in man, but the absolute fall in total plasma ketones which occurred during acetate infusion eliminates this potential mechanism. Similarly, while it has been suggested that the FFA fall associated with exercise is mediated by elevated plasma lactate levels,[19] such a mechanism is not responsible for our findings, since lactate levels were unchanged as FFA fell during acetate infusions, and infusion of lactate itself in man has been shown not to affect plasma FFA.[5]

Growth hormone, administered in pharmacological doses, acutely accelerates peripheral uptake of FFA.[7] The small changes in plasma growth hormone levels observed, coupled with the decline of plasma glycerol levels, are not consistent with such an action in our studies. While ACTH and TSH stimulate triglyceride hydrolysis in vitro, no experimental support exists to suggest that these tropic hormones exert significant fat mobilizing effects as secreted endogenously.[1]

The data permit several positive conclusions. During infusions of 0.45 mmole of sodium acetate per minute, FFA levels decline within 5 minutes. Assuming distribution of acetate within the extracellular space and discounting its utilization,* blood acetate levels could rise no more than 3.0 to 3.5 μg per milliliter in this period. Since normal blood acetate levels in man are 3.0 to 7 μg per milliliter, it seems likely that we are demonstrating a phenomenon of physiological significance. However, since free acetate derived from ethanol oxidation or infused directly may behave quantitatively in a different way from the acetate pool derived from all common nutrient substances,* direct extrapolation of our findings to the physiological events which follow a meal cannot be made.

Nonetheless, the data point to the existence of a previously unknown lipolytic control mechanism in man which operates independently of the measured neurohormonal factors. It is possible that acetate, a lipogenic precursor in adipose tissue,[8] may directly inhibit lipase activity by a negative feedback mechanism analogous to that by which long chain fatty acyl-CoA inhibits acetyl-CoA carboxylase in liver.[8]

The authors are grateful to Dr. Lewis Braverman, who referred the patients with thyrotoxicosis to us, and to Misses Dorothea Barnes and Joan Hacy for skilled technical assistance.

*Lundquist has documented the rapid disappearance of acetate from the circulation after the injection of acetic acid or acetates.[6] Thus, the assumption that no free acetate is utilized in this period will necessarily provide estimates of blood acetate levels in excess of their true values.

*Recently, these studies have been confirmed,[8] and acetate levels measured by gas-liquid chromatography rose with both alcohol and acetate administration.

REFERENCES

1. Carlson, L. A.: Inhibition of the mobilization of free fatty acids from adipose tissue. Ann. New York Acad. Sc. 131: 119, 1965.
2. Friedberg, S. J., Sher, P. R., Rosenoff, M. D., and Estes, E. H., Jr.: The dynamics of plasma free fatty acid metabolism during exercise, J. Lipid Res. 4: 34, 1963.
3. Lieber, C. S., et al.: Effect of ethanol on plasma free fatty acids in man, J. Lab. & Clin. Med. 59: 826, 1962.
4. Jones, D. P., Person, E. S., and Lieber, C. S.: Free fatty acid turnover and triglyceride metabolism after ethanol ingestion in man, J. Lab. & Clin. Med. 66: 804, 1965.
5. Feinman, L., and Lieber, C. S.: Effect of ethanol on plasma glycerol, Am. J. Clin. Nutrition 20: 460, 1967.
6. Abramson, E. A., and Arky, R. A.: Antilipolytic effects of alcohol and acetate in man, Diabetes 16: 508, 1967. (Abst.)
7. Lundquist, P., Tygstrup, N., Mellemgaard, K., and Munck Petersen, S.: Ethanol metabolism and production of free acetate in the human liver, J. Clin. Invest. 41: 955, 1962.
8. Arky, R. A., and Freinkel, N.: Alcohol hypoglycemia. III. Effects of ethanol on plasma

glucose, ketones and free fatty acids in "juvenile" diabetics: A model for "non ketotic diabetic acidosis"? Arch. Int. Med. 114: 501, 1964.

9. Abramsen, E. A., Arky, R. A., and Wachter, K. A.: Effects of propranolol on the hormonal and metabolic responses to insulin induced hypoglycemia, Lancet 2: 1386, 1966.

10. Hohorst, H. J.: L (+) lactate. Determination with lactic dehydrogenase and DPN, *in* Bergmeyer, H. U., editor: Methods of enzymatic analysis, New York, 1963, Academic Press, Inc., p. 266.

11. Bessman, S. P., and Anderson, M.: Estimation of citric acid and ketone bodies by salicylaldehyde-acetone reaction, Fed. Proc. 16: 151, 1957.

12. Snedecor, G. W.: Statistical methods applied to experiments in agriculture and biology, Ames, 1956, Iowa State College Press.

13. Freinkel, N., et al.: Alcohol hypoglycemia. I. Carbohydrate metabolism of patients with clinical alcohol hypoglycemia and the experimental reproduction of the syndrome with pure ethanol, J. Clin. Invest. 42: 1112, 1963.

14. Lipsky, S. R., Alper, B. J., Rabiner, M. E., Van Eck, W. F., and Gordon, M. L.: The effects of alkalosis upon ketone body production and carbohydrate metabolism in man, J. Clin. Invest. 33: 1269, 1954.

15. Lundquist, F., Tygstrup, N., Winkler, K., and Birger Jensen, K.: Glycerol metabolism in the human liver: Inhibition by ethanol, Science 150: 616, 1965.

16. Kipnis, D. M.: Growth hormone and insulin antagonism, *in* Leibel, B. S., and Wrenshall, G. E., editors: On the nature and treatment of diabetes, Amsterdam, 1965, Excerpta Medical, p. 258.

17. Bleicher, S. J., Freinkel, N., Byrne, J. J., and Seifert, H.: Effect of ethanol on plasma glucose and insulin in the fasted dog, Proc. Soc. Exper. Biol. & Med. 115: 330, 1964.

18. Berbencourt, A. V., Mayes, O. E., and Shipp, J. C.: Acetate (-)-(+) metabolism and the effect of acetate on glucose and palmitate metabolism in the perfused rat heart, Metabolism 15: 847, 1966.

19. Gordan, G. J., and Tustison, W. A.: Acetoacetate mediation of the effect of raised arterial glucose upon free fatty acids, Science 146: 770, 1964.

20. Owen, O. E., Morgan, A. P., Kemp, H. G., Sullivan, J. M., Herrera, M. G., and Cahill, G. F., Jr.: Brain metabolism during fasting, J. Clin. Invest. 46: 1589, 1967.

21. Bjjrntorp, P., and Schersten, T.: Effect of beta hydroxybutyrate on lipid mobilization, Am. J. Physiol. 212: 653, 1967.

22. Issekutz, B., Jr., and Miller, H.: Plasma free fatty acids during exercise and the effect of lactic acid, Proc. Soc. Exper. Biol. & Med. 110: 537, 1962.

23. Zierler, K. L., and Rabinowitz, D.: Roles of insulin and growth hormone, based on studies of forearm metabolism in man, Medicine 42: 385, 1963.

24. Astwood, E. B.: The pituitary gland and the mobilization of fat, *in* Renold, A. E., and Cahill, G. F., Jr., editors: Handbook of physiology, Sec. 5, Adipose tissue, Washington, 1965, American Physiology Society, p. 529.

25. Lundquist, F.: Production and utilization of free acetate in man, Nature 195: 579, 1962.

26. Lundquist, F.: The conversion of acetate to blood glucose in [illegible] man, Acta physiol. Scandinav. Suppl. 175: 97, 1962.

27. Wunderad, A. L., and Bondi, A.: Studies on rat [illegible] Effects of insulin on the metabolism of glucose, pyruvate and acetate, J. B. [illegible] 1966.

28. Bortz, W. M., and Lynen, F.: The [illegible] acid CoA derivatives, Biochem. Zeschr. 337: 509, 1963.

29. Crouse, J. R., Gerson, C. D., and Lieber, C. S.: Acetate [illegible] the [illegible] in plasma free fatty acids (FFA) after ethanol, Clin. Res. 15: 149, 1967.

30. Rothlin, M., Rothlin, C. E., and Wendt, V. E.: Fatty acid [illegible] composition in arterial blood, Am. J. Physiol. 203: [illegible] 1962.

Lipid Metabolism: I. Effects of Pressure and Gas Composition on Acetate-C^{14} Incorporation Into Liver Lipids

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Adams, G. M., and S. J. Norton. *Lipid metabolism: I. Effects of pressure and gas composition on acetate-C^{14} incorporation into liver lipids.* Aerospace Med. 42: 146-148, 1971.

The rate of liver lipid biosynthesis was studied as a function of pressure and partial pressure of oxygen. The partial pressure of oxygen was maintained at either 0.2 atm or 1.0 atm in the pressure range of 125 psig to 1000 psig. Liver slices obtained from male Sprague-Dawley rats were incubated in the test environments in Krebs-Ringer bicarbonate buffer containing sodium acetate-C-14. The results obtained indicate that at oxygen partial pressures in the region of 0.2 atm, the rate of incorporation of radiolabel into neutral lipid and phospholipid is pressure-independent; whereas, at oxygen partial pressures near 1.0 atm, the rate of incorporation is pressure-dependent. These results are discussed with respect to their possible metabolic relationships to decompression sickness.

RECENT STUDIES in decompression sickness have introduced the possibility of a physiological-biochemical contribution to decompression sickness that may not be wholly eliminated by physical treatment of the phenomenon. Increasing interest has been centered on the role of blood lipids in the etiology of decompression sickness. Numerous studies have been reported delineating the qualitative and quantitative changes in blood lipids pursuant to decompression sickness.[1-5] Related therapeutic treatments have been devised and appear to minimize both the incidence and severity of decompression sickness in both laboratory animals and man. It seems apparent, however, that effective lipid-oriented therapeutic treatments of decompression sickness are ultimately dependent upon an understanding of the origin of these lipids and of their possible roles in the etiology of decompression sickness. Discussions of the origin of the blood lipid changes have led to the speculation that lipid biosynthesis in the liver, under hyperbaric conditions, may be an important contributing factor.[1]

This study was undertaken to determine if a hyperbaric environment would alter the rate of lipid biosynthesis in an isolated rat liver slice. Thus, the rate of incorporation of radioactive carbon derived from acetate, into liver lipids was studied in a hyperbaric environment of variable pressures and gas composition.

METHODS

Random bred, male, Sprague-Dawley rats, weighing between 250-300 grams, were sacrificed by cervical dislocation. The liver was excised in a +5° C cold room and rinsed with cold 0.85% saline. The main lobe was removed and placed in cold Krebs-Ringer bicarbonate buffer.[6] The central area of the main lobe was sectioned into thin slices (approximately 1 × 3 × 5 mm). The wet slices were weighed to 0.5 mg accuracy and transferred into planchets containing 1.5 ml of Krebs-Ringer bicarbonate at +5° C. Sodium acetate-C^{14} (0.1 ml; sp. act., 56 mC/mmole; 1 × 10^{6} cpm) was added to each planchet. Control slices were maintained in closed glass containers and a Parr Model 4635 high pressure cell was used to provide the hyperbaric environment.

For those runs requiring a pO_2 of about 0.2 atm, the bomb was sealed for nitrogen compression after the planchets were placed in it. In those runs requiring a pO_2 of about 1.0 atm, the planchets were placed in the bomb, the bomb was flushed for 3 minutes with pure oxygen and then sealed for compression with nitrogen. Air controls were used with all runs and pure oxygen controls at ambient pressure were used when 1 atm pO_2 runs were conducted.

After preparation, the vessels were transferred into a stirred water bath at 37° C. The pressure vessel was immediately pressurized to the desired bottom pressure at a rate of 100 psig/min. After 50 minutes at bottom, the vessel was decompressed at a rate of 100 psig/min. Evolution of gas from tissues was not observed in these experiments; media bubbles were observed only after the 750 psig and 1000 psig exposures. Incubation media temperatures were determined in the pressure vessel by fitting a YSI Model 421 thermistor probe through the decompression outlet and monitoring the temperature with a YSI Model 43 telethermometer. In these cases, subsequent decompression was carried out through the gas inlet valve.

After decompression, enzymatic activity was terminated by the addition of 1 ml of 15% (v/v) trichloroacetic acid. Each slice was washed four times with hot distilled water and homogenized in 10 ml of chloroform: methanol (2:1, v/v). The homogenate was filtered and separated into neutral lipid and phospholipid classes on a short silicic acid column.[2] The entire solutions of each class were eventually taken to dryness in scintillation counting vials. Fifteen milliliters of scintillation fluid (Beckman Flouralloy Dry Mix formula TLA in toluene) was added and the vial radioactivity counted on a Beckman liquid scintillation counter. All counts were determined over a 20-minute period or until 1% counting efficiency was reached. All counts were normalized for tissue weight (cpm/gm. tissue). The results are expressed as per cent of the average air control value obtained on

TABLE I

pO_2	Bottom Pressure (psig)	NEUTRAL LIPIDS Mean[b] % Control	S.D.[c]	N.[d]	PHOSPHOLIPIDS Mean % Control	S.D.	N.
0.2	125	138	32	3	78	15	3
0.2	250	112	16	3	96	6	3
0.2	500	103	24	6	102	21	6
0.2	750	94	22	6	92	29	6
0.2	1000	92	21	5	94	11	5
1.0[a]	125	539	18	3	521	72	3
1.0	250	336	55	4	411	37	4
1.0	500	423	67	5	416	85	5
1.0	750	412	147	5	502	118	5
1.0	1000	466	31	4	473	60	4

[a] The mean and standard deviation for oxygen (pO_2 = 1.0 atm ambient pressure controls were 272 ± 102 for neutral lipids and 268 ± 103 for phospholipids.

[b] Mean percent of air control values.

[c] Standard deviation.

[d] The number of individual slices studied. See methods section for more detailed observations.

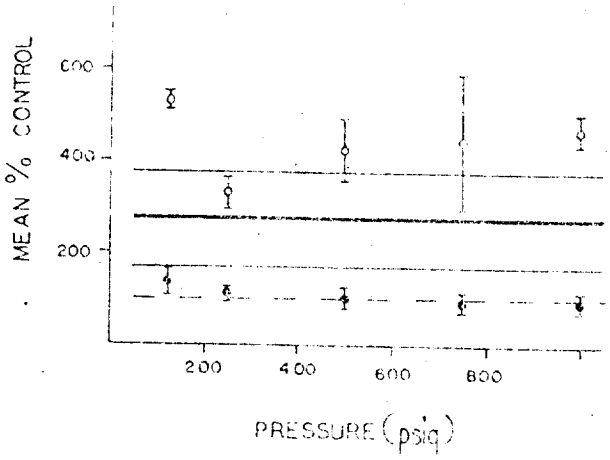

Fig. 1. The incorporation of acetate-C^{14} into total neutral lipids. Broken line, relative air control values; heavy line, mean incorporation; lighter line, S.D. of mean value for 1 atm O_2 at ambient pressure. ●, values obtained at 0.2 atm O_2; ○, values obtained at 1 atm O_2. See methods section for experimental details.

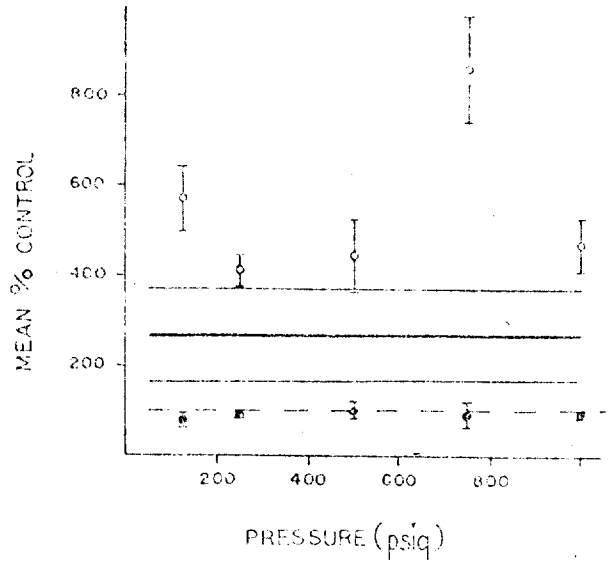

Fig. 2. The incorporation of acetate-C^{14} into total phospholipids. Broken line, relative air control values; heavy line, mean incorporation; lighter line, S.D. of mean value for 1 atm O_2 at ambient pressure. ●, values obtained at 0.2 atm O_2; ○, values obtained at 1 atm O_2. See methods section for further details.

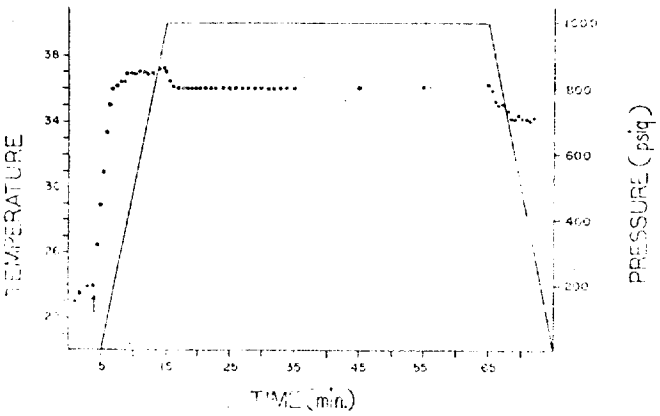

Fig. 3. Media temperature response during a typical 0.2 atm, O_2, 1000 psig run. Broken line, dive profile; dots temperature. C: ↑ pressure cell containing samples placed in temperature regulated water bath.

the same experiment. This method was employed to minimize variability of response between liver tissues from different animals.

RESULTS AND DISCUSSION

The results obtained in this study are shown in Table I. These results indicate that variations in bottom pressures from 125 to 1000 psig have no apparent effect on the incorporation of acetate-C^{14} into either neutral lipid or phospholipid classes. On the other hand, an increase in pO_2 from 0.2 atm to about 1.0 atm has a significant positive effect on the incorporation of radiolabel. Over the range of pressures studied with a pO_2 of 0.2 atm, pressure increases result in an apparent small decrease in the incorporation of label into neutral lipids, as seen in Figure 1; however, this trend is not apparent with the phospholipids at pO_2 of 0.2 atm or in the 1.0 atm pO_2 experiments. Both Figures 1 and 2 denote an increase in label incorporation at a pO_2 of 1 atm above that of the corresponding, pure oxygen, ambient pressure control. This is probably the result of increased media oxygen concentration in the pressurized control. An increase in the media temperature could cause an increase in the rate of label incorporation. The media temperature variations were therefore determined. The results from a typical 1000 psig run are shown in Figure 3. Since under the conditions employed in the study media temperatures do not increase, the observed results must be derived from the increased pO_2 alone.

Between 125 and 1000 psig, the results obtained i this study at 0.2 atm O_2 indicate a pressure-independen rate of lipid biosynthesis. On the other hand, the result indicate a dependence of the rate of lipid biosynthesi on the partial pressure of oxygen. These findings sug gest that altered liver lipid biosynthesis rates are no responsible for the blood lipid changes in the rat pu suant to decompression sickness providing the partia pressure of oxygen is maintained at 0.2 atm throughou the dive. The potential consequences of an increase rate of lipid biosynthesis with increased oxygen partia pressures requires additional study. It may be assume that the observed rate increases with increased pO levels reflect increased fatty acid production. This pro duction could provide a mechanism to enhance glyco lysis at the expense of the pentose phosphate sugars an provide a ready source of glycerol phosphate in th liver. This metabolite would be available for couplin with the fatty acids released by enzymatic mobilizatio of depot lipids resulting in increased levels of comple neutral lipids and phospholipids in the blood. While th explanation represents only one of a myriad of poss bilities, it serves to emphasize the importance of furthe investigation of the oxygen effects observed in this stud

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The experiments reported herein were conducted according the principles enunciated in "Guide for Laboratory Animal F cilities and Care," prepared by the Committee on the Guide fo Laboratory Animal Resources, National Academy of Science National Research Council.

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REFERENCES

1. Adams, G. M., R. E. Danziger, E. M. Neffenger, Jr., T. Saelle and D. E. Crowe: Naval Medical Research Institute Research Report, M4500.02-4010B, Report No 1970.
2. Colodzin, M., E. M. Neffenger, Jr., and H. C. Sweeney: *J. Lipid Res.* 3:251, 1962.
3. Pauly, R. B.: *Can. J. Physiol. Pharm.* 42:849, 1964.
4. Pauly, R. B., C. W. Gowdy and M. Frasse: *Can. J. Physiol. Pharm.* 42:1047, 1967.
5. Pawley, S. M., and A. T. K. Cockett: *Aerospace Med.* 41: 56, 1970.
6. Williams, R. J., and E. M. Lansford, Jr. (Eds.): *The Encyclopedia of Biochemistry.* Reinhold Publishing Corp. New York, 1967, p. 594.

A STUDY OF THE EFFECTS OF GROWTH HORMONE ON FATTY ACID METABOLISM IN VITRO*

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As part of an investigation of regulatory mechanisms in fatty acid metabolism, a study was undertaken of the effects of the growth hormone of the pituitary gland. The well-established ketogenic action of this hormone (1-6), its fat-mobilizing and respiratory quotient-lowering effects (7-12), its growth-promoting activity whereby the protein content of an animal is increased at the expense of body fat (12-14), and its apparent inhibitory action on fatty acid synthesis (15-17) all point to an involvement of this hormone in the metabolism of fatty acids. However, the biochemical sites thereof remain obscure.

The possibility that the growth hormone may exert a direct action on fatty acid catabolism in liver rests on numerous studies in which it was observed that liver preparations of animals treated prior to sacrifice with either growth hormone or anterior pituitary extracts exhibited increases in oxygen consumption or in ketone body formation in the absence or presence of added fatty acids (2, 3, 18-20). In corroboration of this idea, Bondy and Wilhelmi (1) and Tepperman and Tepperman (21) found a decreased ketogenesis in liver slices from hypophysectomized rats. However, the former investigators found that the ketogenesis of surviving liver slices of normal rats was not affected by the growth hormone, whether previously administered *in vivo* or added *in vitro*. The decrease in ketogenesis displayed by liver slices of hypophysectomized rats was attributed by Bondy and Wilhelmi (1) to reduced thyroid function, since this effect was overcome by administration of thyroxine. Shipley (22) found a 46 per cent rise in the endogenous ketone body production of liver slices from fed rats on the addition of anterior pituitary extract in an amount equivalent to 20 mg. of fresh gland. A 27 per cent increase was observed in liver slices from fasted rats. However, Campbell and Davidson (18) found no effect

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† This work will be incorporated in a thesis to be submitted by Arthur Allen to the Graduate Council of Temple University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

of added anterior pituitary preparations on oxygen uptake or acetoacetate production in rat liver slices, and Bondy and Wilhelmi also obtained negative results with crystalline growth hormone (4). Tepperman and Tepperman (21) observed increased ketogenesis in rat liver slices on addition of one growth hormone preparation *in vitro*, but obtained no effects with two other growth hormone preparations.

It is logical to assume that the question of the existence and the nature of a direct effect of growth hormone on fatty acid metabolism can be answered most decisively by studying its action on the catabolism of fatty acids in isolated tissue preparations. Such studies are beset by technical difficulties, however (23). Tissue slices and whole homogenates have a high endogenous oxygen consumption, and, even when an exogenous substrate increases it, there is no assurance that the difference represents the extent of oxidation of the added substrate. Ketogenesis is not a reliable index of fatty acid catabolism, particularly in the tissue slice in which fatty acids may also be converted to CO_2 and to amino acids. Added fatty acids, particularly those of long chain length, exert inhibitory effects on respiration which are difficult to evaluate, and in general it is our experience that responses in the presence of added fatty acids is highly variable.

Some of these difficulties can be overcome by the use of C^{14}-labeled fatty acids. By these means it is possible at least to obtain objective estimates of fatty acid oxidation and of ketogenesis by measuring the incorporation of labeled carbon into respiratory CO_2 and acetoacetate, without the uncertainties inherent in the measurements for endogenous metabolism. In the present report, the results of such studies, described, with liver and homogenates of rat tissues being used, have given no indication that the purified growth hormone exerts any direct effect on the catabolism of fatty acids in rat tissues.

EXPERIMENTAL

The purified growth hormone preparations used in this study were obtained from two sources. The first one was obtained from the Frank W. Horner Company, lot No. C-24-31-O. This preparation, when given to hypophysectomized female rats in a daily dose of 10 γ over a 10 day period, produced an average weight increase of 11 gm.[1] With female rats of 100 gm., according to the assay procedure described by Li *et al.* (22), it exhibited a daily injection of 0.50 mg. over an 18 day period caused about an average weight gain of 1.6 gm. per day as compared with an average gain of 0.25 gm. per day for control animals. The second preparation was obtained from the Armour Laboratories,[2] lot No. M-108.

The carbon-labeled fatty acids were prepared as described previously

[1] L. Nicholl of the Frank W. Horner Company, private communication.

[2] We are indebted to the Armour Laboratories for supplying us with this material.

(23) and the methyl-labeled sodium acetate and the uniformly labeled glucose (glucose-U-C^{14}) were purchased from the Isotopes Specialties Company, Inc., on allocation by the United States Atomic Energy Commission. Before use, these labeled substrates were diluted with unlabeled material to give final activities in the range of 5000 to 20,000 c.p.m., when assayed as barium carbonate according to our standard counting procedure (25). All the experiments, unless otherwise noted, were conducted with approximately 3 month-old male rats from our colony. Experiments with slices were conducted essentially as described by Weinhouse *et al.* (23), and those with homogenates as described by Wenner *et al.* (25), except that cytochrome *c* and fumarate were omitted from the incubation medium. In the small scale experiments, with 50 mg. of tissue, carrier carbonate in an amount of about 0.25 mmole was added to the contents of the center wells to facilitate recovery and assay of respiratory CO_2. Except where indicated otherwise, yields are reported in microatoms of substrate carbon converted to the product in question, calculated as described in earlier publications (25, 26).

Results

Oxidation of Fatty Acid in Liver—In experiments such as the typical ones shown in Table I, the direct addition of growth hormone to Warburg vessels in which palmitate-1-C^{14} was being oxidized by rat liver slices did not result in an increased incorporation of labeled carbon into CO_2 or acetoacetate. No differences were observed in oxygen uptake or in yields of respiratory CO_2 or acetoacetate, the values being similar to values reported previously in similar experiments (23). Hence, to save space, these data generally were omitted from Tables I to V. Radiochemical yields are expressed in microatoms of substrate carbon converted per hour per gm. of dry tissue, the dry weight being estimated as one-fifth of the fresh weight. These conversion values were calculated on the assumption that there is equal utilization of all fatty acid carbons (27). Radiochemical yields of acetoacetate were calculated directly from assays of carboxyl and β-carbon. With palmitate the ratio of β to COOH was 1, and with butyrate it was about 0.8; these ratios were uninfluenced by the addition of the hormone. The small differences between the supplemented and unsupplemented flasks in the utilization of palmitate carbon for CO_2 production or for ketogenesis are not regarded as significant. The conversion of butyrate carbon to CO_2 or to acetoacetate likewise was not affected significantly by the presence of growth hormone. As indicated in Experiment 4, pretreatment of the rat *in vivo* with growth hormone likewise had no effect on the oxidation of palmitate by liver slices.

The withdrawal of a citric acid cycle component via a side reaction might

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result in a deficiency of oxaloacetate for condensation with acetate, whereupon ketogenesis might occur as an alternate metabolic pathway. Ranganath and Peterson (28) showed that the addition of ammonium chloride to rat liver slices increased ketogenesis from pyruvate, and they attributed this effect to stimulation of the amination of α-ketoglutaric acid, thereby withdrawing a potential source of oxaloacetate needed for operation of the

TABLE I

Effect of Growth Hormone on Fatty Acid Oxidation in Rat Liver Slices

Each flask contained 4 to 5 gm. of fresh tissue from fed rats, and where indicated, 5 mg. of purified growth hormone (Armour preparation) suspended in 40 ml. of Ringer phosphate solution, with oxygen in the gas phase. Incubations were carried out for 2 hours except for Experiment 4, which was conducted for 3 hours. Palmitate concentration was 0.0025 M and lactate 0.01 M. The values are given in microatoms of substrate carbon converted to CO_2 or to acetoacetate per gm. of dry tissue per hour. The O₂ values are given in microatoms per gm. of dry tissue per hour.

Exp. No.	Substrate	Oxygen		Respiratory CO_2		Acetoacetate	
		Growth hormone		Growth hormone		Growth hormone	
		Absent	Present	Absent	Present	Absent	Present
		μ-atoms	*μ-atoms*	*μ-atoms C*	*μ-atoms C*	*μ-atoms C*	*μ-atoms C*
2	Palmitate-1-C^{14}	327	313	9.5	7.9	17.3	15.0
	"	332	327	7.1	8.5	15.2	14.5
3	"	331	329	8.1	8.8		
	" + 0.01 M NH_4Cl	331	334	7.0	7.7		
4*	"	343	332	5.7	6.1		
5	Palmitate-1-C^{14}	375	381	69.7	79.2	234	237
	" + 0.01 M NH_4Cl	365	379	67.3	62.5	238	232

* Rats were starved 72 hours prior to experiment.

† Growth hormone injected *in vivo* into female rats pretreated as described for Experiment 2, Table IV.

citric acid cycle. The possibility was considered that the growth hormone might, as a consequence of its growth-promoting action, stimulate amino acid formation from citric acid cycle components, and thus promote ketogenesis. If so, it might be expected that an inhibitory effect of growth hormone on fatty acid oxidation or an enhancement of ketogenesis might be demonstrable in the presence of acetate. However, as denote Experiments 3 and 5 of Table I, neither ketogenesis nor CO_2 production from palmitate or lactate was affected by growth hormone in the presence of ammonium chloride.

Kidney.—The action of growth hormone in kidney is illustrated in Table

II. This experiment is typical of a total of six thus far carried out in which the growth hormone did not enhance fatty acid oxidation. The oxidation of palmitate was depressed somewhat by the growth hormone,[3] the conversion of palmitate carbon to respiratory CO_2 having been reduced from 0.45 to 0.35 microatom. Since kidney homogenates oxidize glucose readily (25), this was included as a substrate. Glucose oxidation was also depressed by the hormone, from 3.2 to 2.1 microatoms. It was interesting to note that, even in the presence of overwhelming quantities of glucose, palmitate oxidation was as high as in the absence of added glucose and this was also true in the presence of added growth hormone. As observed previously

TABLE II

Effect of Growth Hormone on Fatty Acid and Glucose Oxidation in Rat Kidney Homogenate

Each flask contained 50 mg. of homogenized kidney from a fed rat, suspended in 1.5 ml. of isotonic KCl-phosphate solution. Each vessel contained diphosphopyridine nucleotide in a final concentration of 0.002 M and, where indicated, 0.20 mg. of growth hormone. Palmitate concentration was 0.00625 M and glucose 0.005 M. The experiments were conducted 1 hour at 38° with air in the gas phase. The values are given in microatoms of substrate carbon converted to CO_2.

Substrates	Respiratory CO_2	
	Growth hormone	
	Absent	Present
	microatoms C	*microatoms C*
Palmitate-1-C^{14}	0.45	0.35
Glucose-U-C^{14}	3.20	2.10
Palmitate-1-C^{14} + glucose	0.44	0.38
Glucose-U-C^{14} + palmitate	0.41	0.26

(29), the oxidation of glucose was greatly depressed by exogenous palmitate, but the effect was about the same in the presence or absence of added hormone. Thus, in kidney as well as in liver, growth hormone failed to influence either glucose or palmitate oxidation, and also had no effect on the oxidation of one substrate in the presence of the other.

Oxidation of Endogenous Fatty Acids.—To study possible direct effects of

[3] The inhibitory effect of the growth hormone on respiration appears consistently at concentrations greater than 200 γ per ml. and is proportional to the level. This has been observed with dialyzed purified growth hormone and with whole and dialyzed pituitary extracts obtained through the kindness of C. H. Li. The inhibition was not observed with a 10-fold higher concentration of serum albumin but was observed with high concentrations of insulin. This effect, which is receiving further study, does not seem to be of physiological significance, since it is also obtained with heated preparations.

the hormone on fatty acid catabolism under somewhat more physiological conditions, we investigated the oxidation of endogenous palmitate of liver slices, labeled as previously described (29) by prior administration of palmitate-1-C^{14} to the intact rat before sacrificing. As seen in Table III, the only effect of relatively massive doses of the hormone was to inhibit both CO_2 production and ketogenesis from endogenous fatty acids by about one-third.[3] The inhibition was in the total CO_2 and acetoacetate formed and not in the specific activity, indicating that essentially all of the respiration, and not just that of the labeled fatty acids, was inhibited in the growth hormone-treated tissue.

TABLE III

Effect of Growth Hormone on Oxidation of Endogenous Fatty Acids of Rat Liver Homogenate

Each flask contained 500 mg. of homogenized liver obtained from a 24-hour fasted rat suspended in 2 ml. of isotonic KCl phosphate solution, and, where indicated, 16 mg. of growth hormone. Each vessel also contained diphosphopyridine nucleotide in a final concentration of 0.002 M. The experiments were conducted 1 hour at 38° with air in the gas phase. The total activity values are specific activity in counts per minute times micromoles of product carbon.

Additions to Flask	Respiratory CO_2			Acetoacetate		
	Amount	Specific activity	Total activity	Amount	Specific activity	Total activity
	μM	c.p.m.	c.p.m.	μM	c.p.m.	c.p.m.
None	121	120	15,500	13	228	11,300
Growth hormone	76	124	9,400	9	212	7,600

Acetate Metabolism in Normal Rats. In view of the lack of effect of the growth hormone on the catabolism of fatty acids *in vitro*, we turned our attention to its possible effects on lipogenesis. Inasmuch as dietary and hormonal effects on lipogenesis had been observed previously in liver slices incubating C^{14}-labeled acetate (17, 31, 32), this system was employed in the present study. Acetate-2-C^{14} was used, and the effects of prior growth hormone administration on its incorporation into CO_2, acetoacetate, fatty acids, and cholesterol were investigated. The results of these experiments are summarized in Table IV. In general, no appreciable differences were observed in the total amounts of fatty acids and cholesterol isolated from the livers of the three experiments, and this was true also of the total yields of CO_2 and acetoacetate. The values obtained were close to those already reported in similar experiments (30), and hence are not repeated here. With the exception of the glycogen levels and the oxygen uptake values, all the data in Table IV are given in radiochemical yields, expressed as micromoles of acetate carbon converted per gm. of dry tissue per hour.

Inasmuch as the maximal effects of growth hormone on ketogenesis (1–6) and fat mobilization (7–12) were observed within a few hours after its administration, it was felt that any effect on lipogenesis might likewise appear after such an interval. However, it was deemed advisable to test also the effect of prolonged administration.

In Experiment 1, the hormone-treated animals (3 month-old female rats from our stock colony) were given sixteen daily intraperitoneal injections of 0.5 mg. of the Horner growth hormone preparation over 19 days, during which time they exhibited the typical weight-gain response (24). After a subsequent 2 days without the hormone, growth hormone injections were resumed, and at the same time the animals were fasted for 24 hours and then refed for 24 hours with a high carbohydrate diet to insure adequate nutrition (30, 31). They were then given 15 mg. of growth hormone and sacrificed 2 to 3 hours later. Three animals each were used in the treated and control groups, and the design of these experiments followed exactly those previously reported (30, 32). Experiment 2 was conducted essentially in the same way, except that a final dose of only 5 mg. of growth hormone was given 2 to 3 hours prior to sacrifice. In this experiment, one liver from each group was allowed to oxidize palmitate-1-C^{14}, the results with these livers being presented in Table I, Experiment 4. Data on the two remaining livers of treated and control animals are represented as Experiment 2, Table IV.

In Experiments 3 to 5, normal male rats, without pretreatment with the hormone, were fasted and refed as before, and the treated groups were given 5 mg. each of growth hormone 2.5 to 4.5 hours before sacrifice, as indicated in Table IV.

It is apparent that treatment with growth hormone under the conditions employed has no appreciable effect on the glycogen-storing ability or in the respiratory capacity of liver cells. The wide variability in the capacity to incorporate acetate carbon into CO_2 or into acetoacetate makes it difficult to assess the action of the growth hormone on these processes; however, no effects are apparent which cannot be ascribed to variations among animals. The same is probably true of lipogenesis, though there is some indication of a reduced incorporation of acetate carbon into fatty acids. In the first four experiments, the incorporation of acetate into tissue fatty acids was decidedly lower in the hormone-treated animals. The extent of the difference varied from a ratio of treated to untreated slices of 2.5:6.5 = 0.4 in Experiment 1, to 5.9:9.2 = 0.6 in Experiment 2. In Experiment 3, incorporation was decidedly lower than normal in both "treated" and "control" livers, probably a reflection of the shorter refeeding period employed in this experiment. However, in Experiment 5, the picture was reversed; *i.e.*, the incorporation of acetate carbon into tissue fatty acids was almost twice as high in the treated as in the untreated rat liver slices.

TABLE IV

Effect of Growth Hormone Injection on Metabolism of C14-Methyl-Labeled Acetate in Liver Slices

Each experiment included 1 flask of sodium acetate-2-C14 (0.5 gm.) and 2 flasks of liver slices in 50 ml. of Ringer-phosphate-saline solution. The experiments were conducted for 3 hours at 38° C. with oxygen in the gas phase. The values are given in microatoms of acetate carbon consumed per gm. of dry tissue per hour. The glycogen determinations were conducted at the start of the experiments.

Experiment No.	Growth hormone	No. of animals and sex	Glycogen	Oxygen uptake	CO_2	Acetoacetate	Fatty acids	Cholesterol
	mg.		*per cent*	*microliters*	*microatoms C*	*microatoms C*	*microatoms C*	*microatoms C*
1*	15	3 F.	7.1 ± 0.5	313 ± 6	36 ± 5	27 ± 2	2.5 ± 0.9	1.8 ± 0.6
	25	3 "	7.7 ± 0.1	306 ± 10	41 ± 3	16 ± 3	6.5 ± 2.3	0.56 ± 0.01
2	0	3 "	7.2 ± 0.1	278 ± 25	36.6 ± 5.0	9.3 ± 0.8	5.9 ± 3.3	0.73 ± 0.03
	25	2 "	7.1 ± 0.1	253 ± 8	35.1 ± 3.1	15.7 ± 4	9.2 ± 1.3	0.50 ± 0.01
3	0	2 M.†	7.5 ± 0.1	322 ± 21	19.3 ± 1.7	18.4 ± 3.3	0.40 ± 0.16	0.66 ± 0.12
	25	2 "	7.1 ± 0.5	273 ± 23	16.7 ± 6.8	14.0 ± 2.1	1.66 ± 0.33	0.33 ± 0.03
4	0	1 "	9.8	309	27.2	26.0	8.(0)	1.17
	25	1 "	10.3	333	27.9	23.5	11.4	0.81
5	0	2 "	6.6 ± 0.7	341 ± 17	30.8 ± 6.4	21.1 ± 0.6	9.0 ± 1.3	0.85 ± 0.03
	25	3 "	7.8 ± 1.4	306 ± 21	30.5 ± 1.7	20.3 ± 0.5	1.9 ± 1.6	0.81 ± 0.19

* All the rats were fasted for 24 hours and refed for 24 hours prior to sacrifice, except in Experiment 3, where the animals were fasted for 36 hours and refed for 12 hours. In Experiments 1 and 2, in addition to the final injection of growth hormone, animals received 0.5 mg. of growth hormone daily, Monday through Friday, for approximately 3 and 32 weeks, respectively. Growth hormone used in Experiments 1 and 2 was prepared by the Frank W. Horner Company, Lot No. C-24-31-D; growth hormone used in the other experiment was from the Armour Laboratories, Lot No. M-108. Growth hormone was dissolved in physiological saline, pH 8.5, and was injected intraperitoneally. The control animals received saline injections.

† In both Experiments 3 and 5, one of the three livers from the hormone injected animals was lost.

With the exception of Experiment 3, all of the values for incorporation of acetate carbon into fatty acids of "treated" liver are in the range previously observed by us for normal rat liver (30, 32). For this reason, the differences observed in these experiments between "treated" and "untreated" tissues are regarded as resulting from a secondary rather than a primary action of the hormone. Certainly the differences found are of little significance when compared with the 50-fold or greater impairment of lipogenesis occurring after a short fasting period (30, 31).

It has already been pointed out (32) that incorporation data do not necessarily give an adequate representation of fatty acid synthesis, and a procedure has been described for calculating a value which is regarded as closer to the true synthesis rate. This procedure is based on the assumption that the specific activity of the acetoacetate represents that of the acetyl groups undergoing conversion to fatty acids. When lipogenesis was calculated in this manner, the difference between the treated and the control groups was somewhat less than was indicated by the incorporation data. The average value for the ten "treated" livers was 15.0 microatoms as compared with an average of 17.5 microatoms of acetate carbon incorporated per gm. of dry tissue per hour for the twelve control livers.

TABLE V

*Effect of Growth Hormone Injection on Metabolism of Uniformly Labeled Glucose-C14 in Liver Slices**

* The conditions are similar to those reported in Table IV, except for the substitution of 200 μmoles of glucose-U-C14 for the labeled acetate.

Growth hormone injected	Glycogen	Oxygen uptake	CO_2	Acetoacetate	Fatty acids	Cholesterol
mg.	*per cent*	*μmoles*	*microatoms C*	*microatom. C*	*microatom. C*	*microatom C*
5	11.4	250	7.03	0.92	0.54	0.013
5	10.9	287	7.46	0.85	0.73	0.015
0	9.5	263	6.63	0.87	0.37	0.013
0	9.0	273	4.50	0.56	0.20	0.018

* Male rats fasted for 24 hours and refed for 24 hours. The growth hormone was injected intraperitoneally 3 to 4 hours prior to sacrifice (Armour lot No. M-108).

Transcribed in physiological saline, pH 8.5, and was injected intraperitoneally. The control animals received saline injections.

† In both Experiments 3 and 5, one of the three livers from the hormone injected animals was lost.

With the exception of Experiment 3, all of the values for incorporation of acetate carbon into fatty acids of "treated" liver are in the range previously observed by us for normal rat liver (30, 32). For this reason, the differences observed in these experiments between "treated" and "untreated" tissues are regarded as resulting from a secondary rather than a primary action of the hormone. Certainly the differences found are of little significance when compared with the 50-fold or greater impairment of lipogenesis occurring after a short fasting period (30, 31).

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Incorporation into Cholesterol—In the same four experiments in which the growth hormone treatment appeared to reduce the incorporation of acetate into fatty acids, there was an increased cholesterol synthesis. Only in Experiment 5 was there no difference. The average for all of the livers of the treated animals was 1.10 as compared with 0.62 microatom for the controls. The data thus suggest a possible enhancement of cholesterogenesis, but again the results are equivocal because of the variability in the data. It seems clear, however, that, if cholesterol synthesis is stimulated

by the growth hormone, its effect is again not large compared with dietary effects on cholesterol synthesis (32).

Oxidation of and Lipogenesis from Glucose. In Table V are given the results of a similar study in which uniformly labeled glucose-C^{14} was used as the substrate. The animals used in this experiment were part of those used for Experiment 4, Table IV, and were treated exactly as described in Table IV. As was observed previously (32), oxidation of and lipogenesis from glucose in liver slices are low compared with acetate; however, again prior treatment with growth hormone did not influence appreciably the incorporation of glucose carbon into CO_2, acetoacetate, fatty acids, or cholesterol.

DISCUSSION

The results of this study provide no evidence that the growth hormone exerts a direct stimulatory effect on the enzymatic processes of fatty acid catabolism. While this work was in progress, Greenbaum and McLean (2) reported the results of a comprehensive study which led them to conclude that the growth hormone may elicit both an inhibition and a stimulation of the fatty acid oxidase system. These effects occurred, however, only within a 24 hour period after the initial administration of the hormone, and could not be correlated with changes in the respiratory quotient or with the blood and urinary ketone body levels of the intact animals. There are several isolated features of these experiments which, we believe, cast considerable doubt on their validity. The fatty acid oxidase assay system employed by Greenbaum and McLean has a relatively high oxygen consumption in the absence of a fatty acid, and the oxygen consumption due to fatty acid oxidation, as well as the acetoacetate production, is obtained by difference. The assumption that the values thus obtained accurately represent the catabolism of the added fatty acid is probably not warranted. It is probably for this reason that we have adopted the tracer technique, which, by providing an objective, independent estimation of fatty acid catabolism, avoids the uncertainties involved in correcting for endogenous metabolism.

Another uncertainty is introduced, we believe, by the employment in their experiments of succinate in a final concentration of 0.0666 M as a "spark." This means that in each vessel there were present 20 grams of succinate, as compared with 3.0 grams of octanoate or 0.5 gram of glucose. Inasmuch as succinate is readily oxidized in such homogenates, it seems unlikely that fatty acid oxidation can be measured from oxygen uptake alone in the presence of such an overwhelming proportion of competing non-fatty acid carbon. Evidence for a possible inhibitory action of the growth hormone on fatty acid oxidation *in vitro* has already been mentioned,[3] but the physiological significance of this finding is questionable and requires further exploration.

The experiments with labeled acetate as substrate also failed to disclose any effect of growth hormone on the conversion of this metabolic intermediate to CO_2 or to acetoacetate. These findings are consistent with those of Tomkins *et al.* (33) and Baruch and Chaikoff (34), who found no change in liver slices of hypophysectomized rats in the ability to oxidize acetate.

The results of the experiments with labeled acetate suggest that the growth hormone may inhibit the synthesis of fatty acids. This finding would be in accord with several previous studies, but here also the data are not entirely consistent. Welt and Wilhelmi (15) found that the administration of growth hormone depressed the incorporation of deuterium from the body water into liver and carcass fatty acids. Brady *et al.* (16, 17) also made several observations suggestive of an inhibiting action of a pituitary principle on lipogenesis. In cat liver slices, the marked depression brought about by pancreatectomy in the incorporation of acetate carbon into fatty acids was overcome by simultaneous removal of the pituitary, and this effect could be reversed by prior administration of growth hormone. Hypophysectomy caused an increase in lipogenesis in rat liver slices, and, though the addition of growth hormone *in vitro* did not depress incorporation, it did abolish the stimulating effect of insulin addition on fatty acid synthesis *in vitro*. Confirmatory evidence of an inhibition of lipogenesis in liver slices of rats given growth hormone has been supplied by Perry and Bowen (35). However, as in our study, the differences between the control and treated animals were not large, and the percentage difference was lowered on animals fed glucose freely. Apparently the inhibitory effects of growth hormone on fatty acid synthesis in liver slices are relatively minor compared with the effects of short fasting periods or of diabetes (30, 31, 36, 37).

In contrast with the general impression gained by these and other studies just mentioned that the growth hormone inhibits fatty acid synthesis, Baruch and Chaikoff (34) found, surprisingly, that liver slices from hypophysectomized rats largely lost the capacity for fatty acid synthesis. It was found that cholesterol synthesis is also reduced in this condition (33), and this observation is more in line with our finding that the growth hormone somewhat accelerates cholesterogenesis in liver slices. However, it is probably incorrect to expect that the effects of hypophysectomy would necessarily be opposite to those of growth hormone administration.

In summarizing these studies in relation to the results reported by others, it appears to us that there are no grounds at present for the belief that the growth hormone exerts a direct action on the catabolism of fatty acids, either for ketogenesis or for complete oxidation to CO_2. The possibility is

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recognized that conditions *in vitro* may have been unsuitable, and that possible effects were overlooked. However, attempts to demonstrate an effect of the growth hormone on fatty acid catabolism in the intact rat, to be reported separately, have likewise been unsuccessful.

Though some evidence has been brought forward to implicate this hormone in the anabolism of fatty acids, the results of this and other studies make it apparent that further work is required to reconcile conflicting and equivocal results before such an effect is established.

SUMMARY

The effect of the growth hormone on the conversion of C^{14}-labeled fatty acids to carbon dioxide or to acetoacetate was studied in rat tissue slices or homogenates under various conditions. Under no condition were these conversions accelerated by anterior pituitary growth hormone, added *in vitro*, or injected prior to sacrifice of the animals.

Liver slices from rats injected with growth hormone a few hours prior to sacrifice exhibited a somewhat lower incorporation of C^{14}-labeled acetate into fatty acids and an increased incorporation into cholesterol. These effects, however, were neither marked nor consistent.

BIBLIOGRAPHY

1. Bennett, L. L., Kreiss, R. E., Li, C. H., and Evans, H. M., *Am. J. Physiol.*, **152**, 219 (1948).
2. Greenbaum, A. L., and McLean, P., *Biochem. J.*, **54**, 413 (1953).
3. Lotspeich, W. D., and Petersen, V. P., *Am. J. Physiol.*, **176**, 232 (1954).
4. Bondy, P. K., and Wilhelmi, A. E., *J. Biol. Chem.*, **186**, 245 (1950).
5. Campbell, J., Hansler, H. R., Munroe, J. S., and Davidson, I. W. F., *Endocrinology*, **53**, 134 (1953).
6. Carballeira, A., Elrick, H., Mackenzie, K. R., and Browne, J. S. L., *Proc. Soc. Exp. Biol. and Med.*, **81**, 15 (1952).
7. Levin, L., and Farber, R. K., *Recent Progress Hormone Res.*, **7**, 309 (1952).
8. Weil, R., and Ross, S., *Endocrinology*, **45**, 207 (1949).
9. Szego, C. M., and White, A., *Endocrinology*, **44**, 150 (1949).
10. Li, C. H., Simpson, M. E., and Evans, H. M., *Arch. Biochem.*, **23**, 51 (1949).
11. Greenbaum, A. L., and McLean, P., *Biochem. J.*, **54**, 407 (1953).
12. Greenbaum, A. L., *Biochem. J.*, **54**, 400 (1953).
13. Li, C. H., and Evans, H. M., *Recent Progress Hormone Res.*, **3**, 3 (1948).
14. Li, C. H., Simpson, M. E., and Evans, H. M., *Growth*, **12**, 39 (1948).
15. Weil, I. D., and Wilhelmi, A. E., *Yale J. Biol. and Med.*, **23**, 99 (1950).
16. Brady, R. O., Lukens, F. D. W., and Gurin, S., *Science*, **113**, 413 (1951).
17. Brady, R. O., Lukens, F. D. W., and Gurin, S., *J. Biol. Chem.*, **193**, 459 (1951).
18. Campbell, J., and Davidson, I. W. F., *J. Biol. Chem.*, **189**, 35 (1951).
19. Unnor, A. H., *Brit. J. Exp. Path.*, **30**, 389 (1949).
20. Pettoni, A., and Rossi, C. R., *Boll. Soc. chim. biol.*, **33**, 1025 (1951).
21. Tepperman, J., and Tepperman, P. M., *Ann. New York Acad. Sc.*, **54**, 797 (1952).
22. Shipley, R. A., *Am. J. Physiol.*, **141**, 662 (1944).

23. Weinhouse, S., Millington, R. H., and Volk, M. E., *J. Biol. Chem.*, **185**, 191 (1950).
24. Li, C. H., Simpson, M. E., and Evans, H. M., *Growth*, **12**, 15 (1948).
25. Wenner, C. E., Dunn, D. F., and Weinhouse, S., *J. Biol. Chem.*, **205**, 409 (1953).
26. Weinhouse, S., Millington, R. H., and Wenner, C. E., *Cancer Res.*, **11**, 845 (1951).
27. Chaikoff, I. L., Goldman, D. S., Brown, G. W., Jr., Dauben, W. G., and Gee, M., *J. Biol. Chem.*, **190**, 229 (1951).
28. Recknagel, R. O., and Potter, V. R., *J. Biol. Chem.*, **191**, 263 (1951).
29. Allen, A., Friedmann, B., and Weinhouse, S., *J. Biol. Chem.*, **212**, 921 (1955).
30. Medes, G., Thomas, A., and Weinhouse, S., *J. Biol. Chem.*, **197**, 181 (1952).
31. Lyon, I., Masri, M. S., and Chaikoff, I. L., *J. Biol. Chem.*, **196**, 25 (1952).
32. Medes, G., Spirtes, M. A., and Weinhouse, S., *J. Biol. Chem.*, **205**, 401 (1953).
33. Tomkins, G. M., Chaikoff, I. L., and Bennett, L. L., *J. Biol. Chem.*, **199**, 543 (1952).
34. Baruch, H., and Chaikoff, I. L., *Endocrinology*, **56**, 609 (1955).
35. Perry, W. F., and Bowen, H. F., *Endocrinology*, **56**, 579 (1955).
36. Brady, R. O., and Gurin, S., *J. Biol. Chem.*, **187**, 589 (1950).
37. Chernick, S. S., Chaikoff, I. L., Masoro, E. J., and Isaeff, E., *J. Biol. Chem.*, **186**, 527 (1950).

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Fed Reg 27, 6232, (1962)

PART 121—FOOD ADDITIVES

Boiler Water Additives

The Commissioner of Food and Drugs, having evaluated data available to the Food and Drug Administration, and based in part on data submitted by Armour Chemical Division, Armour and Company, 1355 West Thirty-first Street, Chicago 9, Illinois; Betz Laboratories, Inc., Gillingham and Worth Streets, Philadelphia 24, Pennsylvania; Dearborn Chemical Company, 375 Merchandise Mart Plaza, Chicago 54, Illinois; Hagen Chemicals & Controls, Inc., P.O. Box 1346, Pittsburgh 30, Pennsylvania; and Union Carbide Chemicals Company, P.O. Box 65, Tarrytown, New York, and other relevant information, concludes that a regulation should issue concerning safe uses of boiler compounds employed in the preparation of steam that contacts food. Therefore, pursuant to the provisions of the Federal Food, Drug, and Cosmetic Act (sec. 409(c)(1), (d), 72 Stat. 1786, 1787; 21 U.S.C. 348(c)(1), (d), and under the authority delegated to the Commissioner by the Secretary of Health, Education, and Welfare (25 F.R. 8625), Subpart D is amended by adding thereto the following new section:

§ 121.1083 Boiler water additives.

Boiler water additives may be safely used in the preparation of steam that will contact food, under the following conditions:

(a) The amount of additive is not in excess of that required for its functional purpose, and the amount of steam in contact with food does not exceed that required to produce the intended effect in or on the food.

(b) The compounds are prepared from substances identified in paragraphs (c) and (d) of this section, and are subject to the limitations, if any, prescribed:

(c) List of substances:

	Limitations
Ammonium alginate	
Potassium carbonate	
Sodium acetate	
Sodium alginate	
Sodium aluminate	
Sodium carbonate	
Sodium [illegible]	
Sodium hydroxide	
Sodium lignosulfonate	
Sodium nitrate	
Sodium phosphate (mono-, di-, tri-)	
Sodium silicate	
Sodium sulfate	
Sodium sulfite (neutral or alkaline)	
Sodium tripolyphosphate	
Tannin (including quebracho extract)	
Tetrasodium pyrophosphate	

(d) Substances used alone or in combination with substances in paragraph (c) of this section:

	Limitations
Cyclohexylamine	Not to exceed 10 parts per million in steam, and excluding use of such steam in contact with milk and milk products.
Morpholine	Do.
Octadecylamine	Not to exceed 3 parts per million in steam, and excluding use of such steam in contact with milk and milk products.

Any person who will be adversely affected by the foregoing order may at any time within 30 days from the date of its publication in the FEDERAL REGISTER file with the Hearing Clerk, Department of Health, Education, and Welfare, Room 5440, 330 Independence Avenue SW., Washington 25, D.C., written objections thereto. Objections shall show wherein the person filing will be adversely affected by the order and specify with particularity the provisions of the order deemed objectionable and the grounds for the objections. If a hearing is requested, the objections must state the issues for the hearing. A hearing will be granted if the objections are supported by grounds legally sufficient to justify the relief sought. Objections may be accompanied by a memorandum or brief in support thereof. All documents should be filed in quintuplicate.

Effective date. This order shall be effective on the date of its publication in the FEDERAL REGISTER.

(Sec. 409(c)(1), (d), 72 Stat. 1786; 21 U.S.C. 348(c)(1)(d))

Dated: June 22, 1962.

JOHN L. HARVEY,
Deputy Commissioner of Food and Drugs.

[F.R. Doc. 62-6232; Filed, June 22, 1962; 8:48 a.m.]

§ 318.7 Approval of substances for use in the preparation of products.

(a) (1) No chemical substance may be used in the preparation of any product unless it is approved in this part or Part 319 of this subchapter or by the Administrator in specific cases.

(2) No product shall bear or contain any substance which would render it adulterated or which is not approved in this part or Part 319 of this subchapter or by the Administrator in specific cases.

(b) [Reserved]

(c) Under appropriate declaration as required in Parts 316 and 317 of this subchapter, the following substances may be added to products:

(1) Common salt, approved sugars (sucrose, cane or beet sugar), maple sugar, dextrose, invert sugar, honey, corn syrup solids, corn syrup and glucose syrup), wood smoke, vinegar, flavorings, spices, sodium nitrate, sodium nitrite, potassium nitrate, potassium nitrite, and other substances specified in the chart in subparagraph (4) of this paragraph may be added to products under conditions, if any, specified in this part or in Part 317 of this subchapter.

(2) Other harmless artificial flavorings may be added to products with the approval of the Administrator in specific cases.

(3) Coloring matter and dyes other than those specified in the chart in subparagraph (4) of this paragraph, may be applied to products, mixed with rendered fat, applied to natural and artificial casings, and applied to such casings enclosing products, if approved by the Administrator in specific cases. When any coloring matter or dye is applied to casings, there shall be no penetration of coloring into the product. When any coloring matter or dye is added to meat fat shortening containing artificial flavoring, the product shall be packed in conventional, round shortening containers having a capacity no greater than 3 pounds.

(4) The substances specified in the following chart are acceptable for use in the preparation of products, provided they are used for the purposes indicated, within the limits of the amounts stated and under other conditions specified in this part and Part 317 of this subchapter. In addition to the substances listed in the following chart, Part 319 of this subchapter specifies other substances that are acceptable in preparing specified products.

Class of substance	Substance	Purpose	Products	Amount
Anticoagulants	Citric acid	To prevent clotting.	Fresh beef blood	0.2 percent—with or without water. When water is used to make a solution of citric acid or sodium citrate added to beef blood not more than 2 parts of water to 1 part of citric acid or sodium citrate shall be used.
	Sodium citrate.			
Antifoaming agent.	Methyl polysilicone	To retard foaming	Soups	10 parts per million.
			Rendered fats	Do.
			Curing pickle	50 parts per million.
Antioxidants and oxygen interceptors.	BHA (butylated hydroxyanisole)	To retard rancidity.	Dry sausage	0.003 percent based on total weight. (0.006 percent in combination.)
	BHT (butylated hydroxytoluene).	do	do	do (0.006 percent in combination.)
	Propyl gallate	do	do	do (0.006 percent in combination.)
	BHA (butylated hydroxyanisole).	do	Rendered animal fat or a combination of such fat and vegetable fat.	0.01 percent (0.02 percent in combination.)
	BHT (butylated hydroxytoluene).	do	do	do (0.02 percent in combination.)
	Glycine	do	do	do (0.02 percent in combination.)
	Propyl gallate	do	do	do (0.02 percent in combination.)
	Resin guaiac	do	do	do (0.02 percent in combination.)

Class of substance	Substance	Purpose	Products	Amount
	Calcium propionate	To retard mold growth.	Pizza crust	0.32 percent alone or in combination based on weight of the flour used.
	Sodium propionate	do	do	
	Sodium hydroxide	To decrease amount of cooked out juices.	Cured hams, pork shoulder picnics and loins, canned hams and pork shoulder picnics, and products covered by § 319.104(d); chopped ham and bacon.	May be used only in combination with phosphates in ratio of four parts phosphate to one part sodium hydroxide; the combination shall not exceed 5 percent in pickle at 10 percent pump level; 0.5 percent in product.
Phosphates	Disodium phosphate	do	do	5 percent of phosphate in pickle at 10 percent pump level; 0.5 percent of phosphate in product (only clear solution may be injected into product).
	Monosodium phosphate.	do	do	Do.
	Sodium hexametaphosphate.	do	do	Do.
	Sodium tripolyphosphate.	do	do	Do.
	Sodium pyrophosphate.	do	do	Do.
	Sodium acid pyrophosphate.	do	do	Do.
Proteolytic enzymes.	Aspergillus oryzae	To soften tissues	Beef cuts	Solutions consisting of water, salt, monosodium glutamate, and approved proteolytic enzymes applied or injected into cuts of beef shall not result in a gain of more than 3 percent above the weight of the untreated product.
	Aspergillus flavusoryzae group.	do	do	Do.
	Bromelin	do	do	Do.
	Ficin	do	do	Do.
	Papain	do	do	Do.
Refining agents (must be eliminated during process of manufacturing).	Acetic acid	To separate fatty acids and glycerol.	Rendered fats	Sufficient for purpose.
	Bicarbonate of soda	do	do	Do.
	Carbon (purified charcoal).	To aid in refining of animal fats.	do	Do.
	Caustic soda (sodium hydroxide).	To refine fats	do	Do.
	Diatomaceous earth; Fuller's earth.	do	do	Do.
	Sodium carbonate	do	do	Do.
	Tannic acid	do	do	Do.
Rendering agents	Tricalcium phosphate.	To aid rendering	Animal fats	Do.
	Trisodium phosphate	do	do	Do.
Artificial sweeteners.	Saccharin	To sweeten product.	Bacon	0.01 percent.
Synergists (used in combination with antioxidants).	Citric acid	To increase effectiveness of antioxidants.	Lard and shortening	0.01 percent alone or in combination with antioxidants in lard or shortening.
			Dry sausage	0.003 percent in dry sausage in combination with antioxidants.
			Fresh pork sausage	0.01 percent on basis of fat content, in combination with antioxidants.
			Dried meats	0.01 percent on basis of total weight in combination with antioxidants.
	Malic acid	do	Lard and shortening	Do.

Class of substance	Substance	Purpose	Products	Amount
	Monoisopropyl citrate.	To increase effectiveness of antioxidants.	Lard, shortening, oleomargarine, fresh pork sausage, dried meats.	0.02 percent.
	Phosphoric acid	do	Lard and shortening.	0.01 percent.
	Monoglyceride citrate.	do	Lard, shortening, fresh pork sausage, dried meats.	0.02 percent.

[1] These are proprietary products, and a list thereof can be obtained from the Scientific Services, Meat and Poultry Inspection, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Washington, D.C. 20250.

[2] Information as to the specific products for which use of this substance is approved may be obtained upon inquiry addressed to the Scientific Services, Meat and Poultry Inspection, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Washington, D.C. 20250.

(d) No substance may be used in or on any product if it conceals damage or inferiority or makes the product appear to be better or of greater value than it is. Therefore:

(1) Paprika or oleoresin paprika may not be used in or on fresh meat, such as steaks, or comminuted fresh meat food products, such as chopped and formed steaks or patties; or in any other meat food products consisting of fresh meat (with or without seasoning), except chorizo sausage and Italian brand sausage, and except other meat food products in which paprika or oleoresin paprika is permitted as an ingredient in a standard of identity or composition in Part 319 of this subchapter.

(2) Sorbic acid, calcium sorbate, sodium sorbate, and other salts of sorbic acid may not be used in cooked sausage or any other product; sulfurous acid and salts of sulfurous acid may not be used in or on any product and niacin or nicotinamide may not be used in or on fresh product; except that potassium sorbate, propylparaben (propyl p-hydroxybenzoate), calcium propionate, sodium propionate, benzoic acid, and sodium benzoate may be used in or on any product only as provided in the chart in § 318.7 (c)(4) or as approved by the Administrator in specific cases.

[35 F.R. 15556, Oct. 3, 1970, as amended at 36 F.R. 11903, June 23, 1971; 36 F.R. 12003, 12004, June 24, 1971; 36 F.R. 23720, Dec. 14, 1971; 37 F.R. 16387, Aug. 12, 1972; 37 F.R. 23822, Nov. 9, 1972]

AOAC, 1960

13.079 Fat and Fat Number (31)
Official

Slice one loaf of bread, and let dry overnight, or until sufficiently dry to grind. Grind bread to ca size of openings on No. 20 sieve, mix, and transfer 50 g to 600 ml beaker. Add 100 ml H_2O and mix. Add 100 ml HCl, mix, cover, and heat on steam bath 1 hr, stirring well 6 or 7 times. Cool in cold H_2O bath (15° or less) and stir. Add 10 g Filter-Cel, or similar absorbent, stir, and mix completely. Prep. 90 mm büchner as follows:

Place two 9 cm S&S No. 590 filter papers (or equiv.) in funnel and apply suction. Mix 10 g Filter-Cel with 50 ml H_2O and rapidly pour mixt. into funnel. (This should make a smooth, even layer of Filter-Cel over paper, without cracks or openings.) Filter sample immediately. Rinse beaker several times with ice-cold H_2O. Just before filtration is complete, wash sides of büchner with ca 100 ml ice-cold H_2O (or until clear filtrate comes thru). Up to this point do not let pad suck dry. Continue with suction until Filter-Cel pad seems dry. Transfer this mass, without paper from büchner to original beaker. Break up mass with rod, dry (overnight, on steam bath, and then heat in oven at 100° ca 30 min. to remove all moisture (material must be dry or fat results will be low). Break up any lumps.

Prep. large Knorr extn tube of ca 200 ml capacity (glass tubing 5 cm diam., 12 cm high from shoulder to top of tube). Pack tube with asbestos tamped tightly to form pad ca ⅛" thick. Insert stem of tube into 2 hole rubber stopper in filtering bell jar connected to suction thru 2-way stopcock. Place 500 ml erlenmeyer within bell jar so that stem of tube passes thru neck of flask. To cool beaker and contents, add 100 ml ether-petr. ether (1+1) and macerate 3-4 min. against sides of beaker with medium-size, stiff metal spatula. Decant into extn tube. Suck dry. Add 80 ml of the mixed ethers to beaker. Work as before 2 min. Transfer contents of beaker to extn tube, suck dry, and tamp with flat-end stirring rod until all ether is removed. To material in tube add 80 ml of the mixed ethers used just previously to rinse out beaker, mix thoroly with stirring rod few min., let stand 1 min., then suck dry, and tamp material as before. Make 2 addnl extns, turning suction on and off carefully to avoid loss of sample in erlenmeyer. Transfer to 1 L beaker. Evap. on steam bath, completely transfer fat with small quantities of petr. ether to tared 150 ml beaker, carefully evap. petr. ether on steam bath, dry at 100° to constant wt (ca 30 min.), cool, and weigh. Calc. % total fat on H_2O free basis.

Weigh duplicate samples of 1 g within ±.03 g fat into 300 ml Florence flasks and add 4 ml glycerol-soda soln, 26.026 c. Heat flask carefully over asbestos gauze until bubbles start to appear, then hold flask ca 1" over the heated gauze until cloudiness or turbidity disappears and mixt. is perfectly clear. After mixt. first becomes clear, 30-60 sec. addnl gentle heating insures complete saponification. Cool: add few pieces of previously ignited pumice stone, 138 ml CO_2-free H_2O, and 3 ml H_2SO_4 (1+4); and proceed as in 26.027, using same app. Use 0.02N NaOH for titrn. Multiply ml 0.02N NaOH used by 1.1 and divide by wt fat used. Perform blank detn and make correction. Report number of ml 0.02N NaOH/g fat as "fat number."

Acetic and Propionic Acids (32) Official

13.080 PREPARATION OF SAMPLE

(a) *Air-dried bread.* Prep. sample as in 13.076-13.077.

(b) *Fresh bread.* For analysis of fresh product, which may be difficult to air-dry without spoilage or loss of volatile acids, pass sample thru meat grinder equipped with ⅛" hole plate, and divide finely by rubbing thru No. 8 sieve. Proceed with analysis promptly (24-48 hr) or preserve with $CHCl_3$ as follows:

To prepd bread in Mason jar filled to ¾ capacity, add washed $CHCl_3$, absorbed in ca 1 g sorbon, ca 5 ml $CHCl_3$ per container. Close jar tightly (self-sealing lids are recommended) and roll to mix contents thoroly. Store samples at ca 25° or refrigerate where higher temp. could

13.081 REAGENTS—See 18.016

13.082 APPARATUS

(a) *Distillation apparatus.* Use volatile acid app., 18.015, or gas-fired steam generator.

(b) *Chromatographic tubes.* Approx. 15×250 mm or ca 15×450 mm, constricted at lower end to ca 1 mm i.d.

(c) *Test tubes.* Approx. 16×150 mm size.

(d) *Eyedropper pipet.* Approx. 180 mm long.

13.083 DISTILLATION

Transfer 10 g of the air-dried bread or 15 g of the prepd fresh bread to 150 ml flat-bottom flask. Add 50 ml H_2O and 10 ml ca 1N H_2SO_4. Mix thoroly and add 10 ml 20% phosphotungstic acid soln. Mix by swirling and add 16 g $MgSO_4 \cdot 7H_2O$. Swirl again to partially dissolve salt. Mixt. should be acid to congo red paper. If not, add few drops H_2SO_4 (1+1). Connect to steam generator. Heat contents on asbestos to boil. Distill and distill 200 ml in 35-40 min. [illegible] comes just before boiling [illegible] to prevent splashing of sample into [illegible] Keep [illegible] and to inlet of steam to tube.

Transfer distillate to 400-600 ml beaker, add ca 10 ml or 0.01 N *Ba(OH)₂ soln*, make alk. to phthln with ca 1 N NaOH, and evap to ca 5 ml. Transfer to 25-30 ml glass test tube, rinse beaker with 3 portions H_2O. If good material adheres, add few drops of ca 1 N H_2SO_4 with final rinse. Make alk. to phthln and evap just to dryness by inserting tube in steam bath or in boiling H_2O. (Air jet hastens evapn.) Det. acetic and propionic acids in evapd distillate by the sepn technic, **18.021(b)(2)** after prepg, testing, and stdzg column as in **13.084**.

13.084 CHROMATOGRAPHIC SEPARATION

(a) *Preparation of partition column.* See **18.021(a)**. (Where amount of propionic acid approaches 20 mg in column and definite band is observed below propionic acid band, use the long chromatographic tube (450 mm) and ca 40 g silicic acid. Then take twice amounts of H_2O, indicator, and NH_4OH as used for 5 g silicic acid.)

(b) *Test of silicic acid for suitability and standardization of column.* Prep. stock solns of formic, acetic, and propionic acids (reagent grade) by dilg 5 ml acid to 250 ml and stdzg the acetic and propionic acids as in **18.021(b)**.

Prep. following dil. stock solns from the stock solns and boiled H_2O: Formic acid, 10 ml to 50 ml; acetic acid, 20 ml to 50 ml.

Prep. following trial mixts of formic acid and known amounts of acetic and propionic acids:

ACIDS AND WATER	MIXTURE A (STOCK SOLNS)	MIXTURE B (DIL. SOLNS)	MIXTURE C
	ml	*ml*	
Formic	10	10	1 ml dil. stock soln
Acetic	10	10	1 ml dil. stock soln
Propionic	10	20	1 ml stock soln
Water	20	None	None
Total vol.	50	50	3 ml
Test aliquot	1	1	3 ml

(Above mixts cover range of acetic and propionic acids usually present when 15 g fresh bread preserved with propionate is used as initial sample.)

Pipet indicated test aliquots from mixts *A* and *B* and entire mixt. *C* into bottom of glass test tubes (16×150 mm), neutralize with ca 1 N $NaOH$, using phthln, and add 1 drop excess.

Proceed with evapn, sepn, and titra as in **18.021(b)(1)** and (2). Calc. results for acetic and propionic acids to mg/100 g sample. Following factors are based on 15 g fresh bread or 10 g air-dried bread:

Fresh bread (mg/100 g):

Acetic acid = 4.00 × ml 0.01 N $Ba(OH)_2$

Propionic acid = 4.93 × ml 0.01 N $Ba(OH)_2$

Air-dried bread (mg/100 g):

Acetic acid = 6.00 × ml 0.01 N $Ba(OH)_2$

Propionic acid = 7.40 × ml 0.01 N $Ba(OH)_2$

(c) *Identification of acids.* See also **18.021(c)**. With trial mixts *A*, *B*, and *C* and with most breads to which propionate has been added, only 3 definite bands appear on column and they elute in this order: propionic, acetic, and formic acids. Method provides for addn of enough formic acid to supply definite following band to insure that acetic acid is completely related. Amount of mobile solvent required to move each acid from top of column to point of emergence is function of concn of that acid. For amounts of acetic acid normally present in bread, differences in threshold vol are not critical. To identify propionic acid by threshold vol., however, it is necessary to check threshold vol. for the concn indicated by titra. Sepn of mixts *A*, *B*, and *C* supply enough data for amounts normally found. Threshold vols may be predicted for intervening concns by plotting concn against detd threshold vols.

Due to differences in propionic threshold vol., make change in mobile solvents from 1% butanol-$CHCl_3$ to 10% butanol-$CHCl_3$ at a constant vol. of 1% butanol-$CHCl_3$ rather than at propionic acid threshold, preferably when greater part of propionic acid has been eluted. Then acetic acid threshold vol. will not be affected by changes in propionic acid threshold vol.

Acids as sepd in butanol-$CHCl_3$ solns may be further identified by formation of mercurous acetate or mercurous propionate crystals (*33*).

13.085 **Citric Acid** (*33*) **First Action**

To wt air-dried bread equiv. to 77.7 g moisture-free bread, in 500 ml vol. flask, add 400 ml *alc. H_2SO_4-phosphotungstic acid soln* (25 ml 1 N H_2SO_4, 20 ml 20% phosphotungstic acid soln, and 55 ml H_2O, dild to 500 ml with alcohol), shake 5 min., dil. to mark with the H_2SO_4-phosphotungstic acid soln, and let stand overnight. Readjust to mark with alcohol, shake 5 min., and filter with suction on paper in 12 cm Büchner. Transfer 325 ml filtrate to centrifuge bottle, add 30 ml *$PbOAc_2$ soln* (75 g of the salt + 1 ml HOAc dild to 250 ml with H_2O), shake 5 min., and centrifuge at ca 900 rpm 15 min. Decant supernatant (disregard turbidity), let drain, transfer residue with ca 150 ml H_2O to 250 ml vol. flask, and thoroly sat. with H_2S. Dil. to mark with H_2O, shake thoroly, and filter thru large folded paper. Proceed as in **20.049**, using 200 ml aliquot.

13.086 **Ash** (*35*) **Official**

Use 3-5 prepd sample, **13.076**, and proceed as in **13.005** or **13.008**.

[illegible] bottle stand until clear. If [illegible] emulsion forms, pour off from Mojonnet flask as much of ether fat soln as possible into tared flask, stand, add 1–2 ml alcohol to Mojonnet flask, swirl, and again let mixt. sep.

Rapid Modified Babcock Method (11)—First Action

(For canned fish)

18.013 DETERMINATION

Weigh 5 g ground and mixed sample into Paley-type Babcock cheese bottle (Kimble Glass No. 508, 20% size), stopper, and add ca 30 ml of reagent prepd by mixing equal vols HOAc and 70–72% $HClO_4$. Place in boiling H_2O bath (stainless steel beaker preferred), swirling occasionally until digestion is complete (ca 20 min.). Remove from bath, add reagent until fat is well up in calibrated neck of bottle, centrifuge 2 min. at ca 600 rpm, and read % fat with dividers. If fat falls below calibration, add more reagent, centrifuge 1 min., and read again.

18.014 Fat in Fish Meal—First Action
See 22.037

Volatile Fatty Acids (*12*)—Official

18.015 APPARATUS

(a) *Steam distillation assembly*—Fig. 31. Assembly consists of boiler flask, 3 L, giving steam at constant rate so as to produce constant rate of distn, distn flask, condenser, and 200 ml vol flasks as receivers. Std distn flask with side arm (ca 9 mm o. d.) attached near center of neck, and with steam inlet tube (ca 10 mm o. d.), is satisfactory. Heating coil of steam generator is made by winding 5 feet 28 gauge Chromel wire (or equiv.) around hollow pipe ca 0.25" diam. and heating red hot to detemper wire. Leads into boiler flask are brass, Cu, or other non-ferrous metal ca 3/32" diam.

Any similar distn assembly may be used if it is of capacity to handle vols specified in method and gives 57 ± 2% recovery of acetic acid on distn.

(b) *Chromatographic tube.*—Approx. 15×250 mm.

(c) *Source of air pressure or compressed N gas equipped with pressure regulator.*—If such source is not available, following system serves purpose: Fit 1 L side arm flask with 2 hole rubber stopper. Pass glass manometer tube 70 cm long thru one hole in stopper so that it reaches bottom of flask, and thru other hole pass glass tube ca 8 cm long, whose upper end is connected to top of chromatographic tube by rubber tubing. Connect rubber hand-aspirator bulb to side arm of flask. Fill flask with Hg to depth of 1.5 cm. Height of Hg column in manometer tube indicates pressure in system. 25 cm (equiv. to ca 5 lbs.) To maintain reservoir pressure when chromatographic tube is disconnected, fit stopcock into line leading from flask to

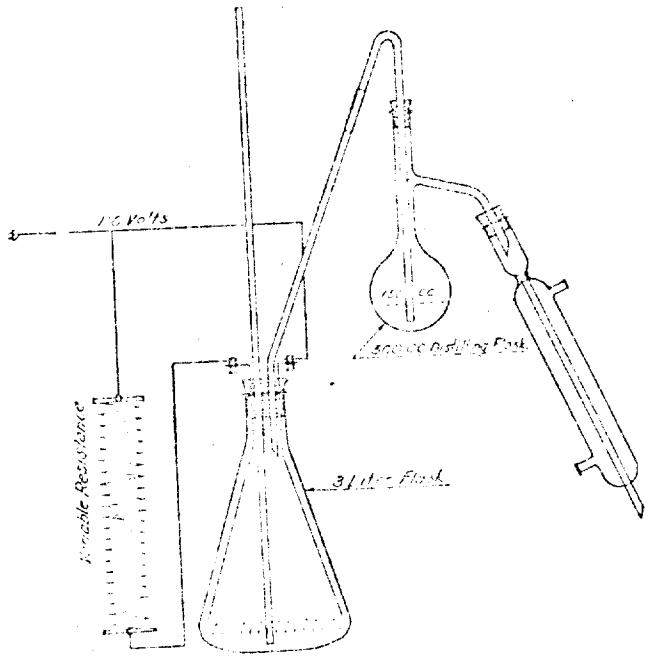

FIG. 31. STEAM DISTILLATION ASSEMBLY

chromatographic tube; and to prevent valve in hand aspirator from leaking, insert second stopcock between side arm and bulb.

(d) *Rubber bulb.*—5 ml. (Type used on dropping bottles.)

18.016 REAGENTS

(a) *Butanol in chloroform.*—1%. Remove alcohol from USP $CHCl_3$ by washing 3 times with ½ vol. H_2O. Add 10 ml *n*-butanol to 1 L washed $CHCl_3$ in separator, shake vigorously, add 25 ml H_2O, and shake again. Let lower layer stand until clear, and drain. Discard aq. layer.

(b) *Butanol in chloroform.*—10%. To 900 ml USP $CHCl_3$ (*not* previously washed) in separator add 100 ml *n*-butanol, shake vigorously, add 25 ml H_2O, and shake again. Let $CHCl_3$ stand until clear, and drain. Discard aq. layer.

(c) *Alphamine Red R indicator.*—Dissolve 50 mg mono-NH_4 salt 3-(4-anilino-1-naphthylazo)-2,7-naphthalene disulfonic acid (Eastman No. 6410) in 25 ml H_2O. (Red color produced by 1 drop indicator in 20 ml H_2O must be changed to violet by 1 drop 0.01*N* HCl.

(d) *Cresol red indicator.*—Dissolve 50 mg *o*-cresolsulfonphthalein in 20 ml alcohol, add 1.3 ml 0.1*N* NaOH, and dil. to 50 ml with H_2O. Use 2 drops for each 25 ml aq. soln.

(e) *Barium hydroxide soln.*—0.01*N*. (Store in paraffin-lined bottle and protect from CO_2 of atmosphere with soda-lime or Ascarite; dispense from 10 ml buret.) (0.01*N* NaOH may be used instead of $Ba(OH)_2$ except in titrn of Soln *B*, **18.019**. NaOH soln must be used in titrn of Soln *A*, **18.019**, since Na salts are required for chromatography.)

(f) *Sodium acetate-sodium chloride soln.*—Dissolve 12 g NaCl and 25 g NaOAc.$3H_2O$ in H_2O and dil. to 500 ml.

(g) *Silicic acid.*—Reagent grade "100-mesh" powder, suitable for chromatography (Mallinckrodt Chemical Co. No. 2847 or equiv.).

18.017 STANDARDIZATION OF DISTILLATION APPARATUS

Place app., **18.015**(a), in laboratory so that it is free from drafts and sudden changes in temp. Make mark on boiler flask at 1.5 L level, fill to this mark with H_2O, heat to boiling, and boil several min. before starting distn. Transfer 150 ml H_2O to distn flask, add 1 drop H_2SO_4 (1+1), connect condenser, insert steam inlet tube into distn flask, and bring contents of flask to incipient boiling with burner. Connect steam inlet tube with steam supply from boiler, and steam distill. Regulate rate of evolution of steam and height of small flame of burner under distn flask so that vol. of liquid in distn flask is kept constant at 150 ml and distillate collects at rate of 200 ml hr. (Period of collection may vary 5 min. for 200 ml of distillate. The 150 ml vol. in distn flask should remain constant within ±10 ml. Boiling may be stopped to permit test of constancy of 150 ml vol. by momentarily interrupting steam supply. Few trials will show conditions necessary to maintain constant vol. in distn flask and constant rate of distn.) Det. blank on 2 successive 200 ml portions of distillate by titrg with 0.01*N* alkali (phthln) in CO_2-free atmosphere.

Transfer 50 ml ca 0.1*N* HOAc (conen must be accurately known) to distn flask, add 1 drop H_2SO_4 (1+1) (avoid contact with neck of flask) and 100 ml H_2O. Collect 200 ml distillate and titr. with 0.1*N* alkali. Correct for titrn blanks and compute % acid distd. Distn technic and app. are satisfactory when recovery is 57 ± 2%. App. so adjusted gives distn rates on propionic and butyric acids of 81% and 92% (± 2%), resp. (*B*), on 200 ml distillate.

18.018 PREPARATION OF SOLUTION

Comminute sample (include entire contents of canned products) by passing 3 times thru food chopper, mixing after each grinding. Weigh 50 g comminuted material into tared 500 ml wide-mouth erlenmeyer, add ca 150 ml H_2O, stopper flask, and shake vigorously ca 1 min. to effect thoro suspension of material. Add 25 ml 1*N* H_2SO_4, mix, ppt proteins with *20% phosphotungstic acid soln* (40 ml is usually enough), make to 300 g with H_2O, shake vigorously ca 1 min., and filter thru 24 cm rapid folded paper.

18.019 DISTILLATION AND COMPUTATION OF VOLATILE ACID NUMBER

Pipet 150 ml prepd soln into distn flask of app. and make acid to *Congo red pap.* with H_2SO_4 (1+1). Steam distill as in **18.017**. Collect 200 ml distillate, titr. with 0.01*N* NaOH, and designate as *A*. Collect second 200 ml portion distillate, titr. with the 0.01*N* $Ba(OH)_2$ soln, and designate as *B*. To calc. volatile acid number multiply titrn obtained on distillate *A*, corrected for blank, by 4.

DETERMINATION OF INDIVIDUAL VOLATILE FATTY ACIDS

18.020 *Formic Acid*

Add 2 drops satd $Ba(OH)_2$ soln to distillate *B*, **18.019**, and evap. to dryness on steam bath. Add ca 5 ml H_2O to residue and 1 ml more of 1*N* HCl than necessary to liberate volatile acids. Filter thru small paper into 125 ml erlenmeyer with $ pint and wash paper with H_2O [illegible] that total filtrate equals 20–40 ml. Add 10 ml of the NaOAc–NaCl soln and 10 ml [illegible] H_2O [illegible]. Connect flask with $ [illegible] steam bath 2 [illegible]

With suction thru glass siphon attached to funnel by rubber stopper, transfer ppt of Hg_2Cl_2 to previously weighed ACS-type microfunnel having coarse glass disk provided with mat of asbestos ca 2 mm thick. Rinse flask with H_2O followed by alcohol. Dry 30 min. at 100°, cool, and weigh. Weigh funnel with another funnel, prepd with asbestos and treated similarly as one contg ppt. as counterpoise.

Wt Hg_2Cl_2 (mg) ×0.0975 = mg formic acid in distillate. To calc. total formic acid originally present in aliquot of sample in distn flask before distn, divide mg formic acid found by 0.24 (fraction formic acid distd in second 200 ml distillate) and multiply by 4 to obtain formic acid in 100 g sample being analyzed.

18.021 *Chromatographic Separation of C_2 to C_4 Saturated Fatty Acids*

(a) *Preparation of partition column.* To 5 g silicic acid in mortar add 1 ml of the Alphamine Red R indicator soln and enough 1N NH_4OH to give alk. color of the indicator (1 drop is usually enough). Add max. quantity of H_2O that the silicic acid will hold without becoming sticky or agglomerating in the butanol-$CHCl_3$ soln. (This quantity must be detd for each batch of silicic acid and usually varies from 50-75% of wt of silicic acid.) Mix thoroly with pestle until homogeneous. Add few ml of the 1% butanol in $CHCl_3$, mix to form paste, and then add enough solvent to form slurry that pours readily. Pour this slurry into chromatographic tube contg small cotton plug in neck of constricted end. To avoid air pockets tilt tube slightly while pouring. If air bubbles form while pouring, eliminate by stirring suspension in tube with long glass rod.

Clamp tube vertically in ring stand. In top insert 1 hole rubber stopper fitted with glass tube bent to 90° angle and held in place by Bunsen clamp against pressure to be exerted. Connect bent glass tube to pressure source, 18.015(c). Adjust pressure to 5-10 lb/sq. in., so that excess solvent is forced thru column dropwise.

During removal of excess solvent the gel packs down. As column packs down, particles of gel adhere to wall of the tube, but eventually gel leaves wall of tube relatively clean. This is point of optimum density for column, and column is ready for use. Do not let column dry below surface of the gel, as such drying or "cracking" renders column useless. If column cracks before acids are added, gel can be extruded from tube, reslurried with solvent, and again poured into tube. If gel is not packed evenly or if air pockets are present, jagged fronts may occur where soln passes such points.

(b) *Test of silicic acid for suitability and standardization of column.* (1) *Preparation of known acid mixt.*—Pipet 1 ml of each of following acids into sep. 50 ml vol. flasks: Formic, acetic, propionic, butyric, and valeric. Dil. to vol. with H_2O, and mix to prep. stock solns of these acids. Pipet 10 ml of each stock soln into a 125 ml erlenmeyer to prep. acid mixt. to be used for stdzn. Keep flask tightly stoppered.

Pipet 1 ml of each stock soln, except formic acid, into sep. erlenmeyers, dil. to ca 15 ml with boiled H_2O, and titr. with the 0.01N alkali, using cresol red indicator. End point is reached when soln assumes pink color that persists ca 45 sec. During titrn bubble stream of CO_2-free air or N thru soln. (CO_2-free air can be prepd by bubbling air thru layer of 20% NaOH soln at least 8 cm deep. Trap or cotton filter plug between wash soln and titrn flask prevents contamination by droplets of the NaOH soln. Satisfactory gas washing bottle can be made from 2 L Florence flask fitted with 2 hole rubber stopper.) To det. quantity of each acid present in 1 ml of mixt. divide quantity of each acid in 1 ml of its stock soln by 5.

Pipet 0.1 ml of the acid mixt. into 50 ml beaker, neutralize with 1N NaOH, using phthln, and add 1 drop NaOH soln in excess. Evap. to dryness on steam bath.

(2) *Separation technic.* (Good sepn and yields depend upon transfer of sample to column with quantities of solvent specified.) To dry residue of Na salts add 2 ml of the 1% butanol in $CHCl_3$ soln and 3 drops H_2SO_4 (1+1), and stir with glass rod until all Na salts are converted to free acids (acid to Congo red paper). Add anhyd. Na_2SO_4 in 0.2 g portions until aq. phase is absorbed. Place 50 ml graduated cylinder under prepd column to catch foreruns, vol. of which is threshold vol. for valeric acid.

Decant supernatant (or use eyedropper pipet) onto column, pouring it slowly down side of tube in order to keep surface of gel level. Apply pressure until solvent just disappears into gel. Wash beaker with 1 ml of the solvent, pour onto column, and with stirring rod transfer residue in beaker to column. Wash beaker with another 1 ml solvent, transfer to column, wash inside of tube with 1 ml solvent, and apply pressure until solvent just disappears into gel. Fill tube with solvent and apply pressure. Change receiver (graduated cylinder) each time lower edge of a band reaches point 2-5 mm above narrowest portion of constriction of tube. (Acids elute in following order: 1, valeric; 2, butyric; 3, propionic; and 4, acetic.) Record foreruns vol., also vol. of solvent required to elute each of remaining acids, except formic. Do not try to elute formic acid band. After propionic acid (third band) reaches constriction of tube, unclamp and pour off remaining solvent in chromatographic tube and fill tube with the 10% butanol in $CHCl_3$.

Det. threshold vol. of butyric acid by adding forerun vol. (valeric threshold vol.) to vol. of solvent required to elute the valeric acid. To obtain threshold vol. of propionic acid add threshold vol. of butyric acid to vol. of solvent required to elute butyric acid. In similar manner obtain threshold vol. of acetic acid.

To compare threshold vols, when different quantities of acids are present, repeat stdzn, using 1 ml of the acid mixt. (ca 4 mg of each acid), but change solvent in chromatographic tube to 10% butanol in $CHCl_3$ when vol. of eluate equiv. to threshold vol. found for ca 0.5 mg propionic acid in first stdzn has been collected.

Transfer eluates to sep. 125 ml erlenmeyers, rinsing each graduated cylinder with three 5 ml portions of H_2O. Add 1 drop of the cresol red indicator soln and titr. with 0.01N alkali in CO_2-free atmosphere, as above. However, as end point approaches, stopper flask and shake vigorously to completely ext. acids from solvent phase. Correct titrn of each eluted band for blank as follows: Collect 25 ml of the butanol-$CHCl_3$ mixt. from column before any acids are transferred, add 15 ml boiled H_2O, and titr. as above with 0.01N alkali.

If bands are not clearly differentiated or recoveries are <90%, reject the silicic acid. (Addnl stdzn with respect to threshold vol. may be desirable for identification in some instances, (c).)

(c) *Identification and determination.*—Evap. distillate A obtained in **18.019** to small vol., transfer to 50 ml beaker, and proceed exactly as in (b)(2). When vol. eluate equiv. to threshold vol. found for 0.5 mg propionic acid in stdzn of column collects, change solvent in tube to the 10% butanol in $CHCl_3$ as above even if no propionic acid is present. Do not change receiver until lower edge of next band reaches point 2–5 mm above narrowest portion of constriction of tube, or until titrn of 5 ml fractions approaches that of the blank.

Identify acids by comparing their threshold vol. with those for ca same quantities and ratios of known acids found in stdzn procedure. Threshold vol. of given quantity of each fatty acid is characteristic and quite reproducible under similar conditions. However, if conditions change, such as by use of different batch of silicic acid or different quantity of same batch, or different quantity of H_2O, threshold vol. for each acid must be redetd. (If present, *iso*-butyric acid is measured as *n*-butyric acid.) For further identification as characteristic salts see reference (*1*).

To calc. quantities of acetic, propionic, and butyric acids in terms of ml 0.01N alkali in aliquot, divide titrns by 0.57, 0.81, and 0.92, resp.; multiply these values by 2.40, 2.96, and 3.52, resp., to obtain mg of each acid/100 g sample.

Histamine-Like Substances

Biological Method (15)—Official

18.022 APPARATUS

(a) *Kymograph.*—Kymograph with horizontal muscle lever arm having friction or gravity writing point.

(b) *Muscle bath.*—At least 50 ml capacity, surrounded by 37° constant temp. bath. *See* Fig. 32. May be conveniently filled and emptied thru 3-way stopcock: 1 tube connected to reservoir of Ringer-Locke soln thru bulb or coil immersed in bath; other tube connected to vac. thru suction flask as waste receiver. Bubble air, filtered thru cotton, slowly and continuously around intestine from fine capillary tube.

18.023 REAGENTS

(a) *Sodium chloride stock soln.*—180 g/L.

(b) *Potassium chloride stock soln.*—42 g/500 ml.

(c) *Sodium bicarbonate stock soln.*—15 g/500 ml.

(d) *Atropine sulfate stock soln.*—1.0 g/500 ml.

(e) *Calcium chloride stock soln.*—24 g anhyd. salt/500 ml.

(f) *Ringer-Locke soln.* NaCl, 0.9%; KCl, 0.042%; $CaCl_2$, 0.024%; $NaHCO_3$, 0.015%; dextrose, 0.1%; and atropine sulfate, 0.001%. Add 100 ml (a) and 10 ml (b), (c), and (d) to 2 L vol. flask. Add H_2O to vol. of ca 1800 ml and then 10 ml (e) while swirling. Add 2 g anhyd. dextrose before use. Dil. to vol. with H_2O. Keep soln contg dextrose in refrigerator when not in use, discarding when it becomes moldy.

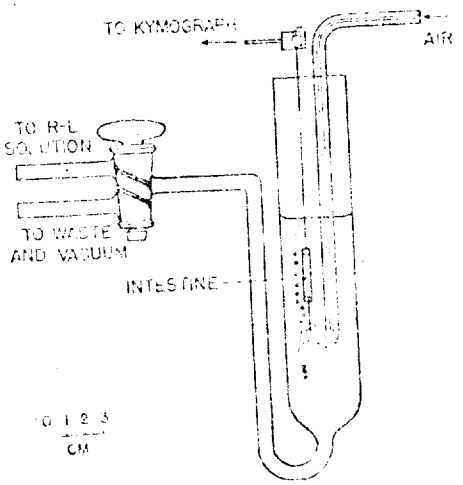

FIG. 32. [illegible]

AOAC, 1965

13.076 **Fat and Fat Number (31)**
Official, Final Action

Slice one loaf of bread, and let dry overnight, or until sufficiently dry to grind. Grind bread to passing openings on No. 20 sieve, mix and transfer 50 g to 600 ml beaker. Add 100 ml H_2O and mix. Add 100 ml HCl, mix, cover, and heat on steam bath 1 hr, stirring well 6 or 7 times. Cool in cold H_2O bath (25° or less) and stir. Add 40 g Filter-Cel, or similar absorbent, stir, and mix completely. Prep. 90 mm Buchner as follows:

Place two 9 cm S&S No. 590 filter papers (or equiv.) in funnel and apply suction. Mix 10 g Filter-Cel with 50 ml H_2O and rapidly pour mixt. into funnel. (This should make a smooth, even layer of Filter-Cel over paper, without cracks or openings.) Filter sample immediately. Rinse beaker several times with ice-cold H_2O. Just before filtration is complete, wash sides of Buchner with ca. 100 ml ice-cold H_2O for until clear filtrate appears then. Up to this point do not let pad suck dry. Continue with suction until Filter-Cel pad seems dry. Transfer this mass, without paper, from Buchner to original beaker. Break up mass with rod, dry (overnight) on steam bath, and then heat in oven at 160° ca 30 min. to remove all moisture (material must be dry or fat results will be low). Break up any lumps.

Prep. large Knorr extn tube of ca 200 ml capacity (glass tubing 5 cm diam., 12 cm high from shoulder to top of tube). Pack tube with asbestos tamped tightly to form pad ca 3" thick. Insert stem of tube into 2 hole rubber stopper in filtering bell jar connected to suction thru 2-way stopcock. Place 500 ml erlenmeyer within bell jar so that stem of tube passes thru neck of flask. To cool beaker and contents, add 100 ml ether-petr. ether (1+1) and macerate 3-4 min. against sides of beaker with medium-size, stiff metal spatula. Decant into extn tube. Suck dry. Add 80 ml of the mixed ethers to beaker. Work as before 2 min. Transfer contents of beaker to extn tube, suck dry, and tamp with flat-end stirring rod until all ether is removed. To material in tube add 80 ml of the mixed ethers used just previously to rinse out beaker, mix thorely with stirring rod few min., let stand 1 min., then suck dry, and tamp material as before. Make 2 addnl extns, turning suction on and off carefully to avoid loss of sample in erlenmeyer. Transfer to 1 L beaker, evap. on steam bath, completely transfer fat with small quantities of petr. ether to tared 150 ml beaker, carefully evap. petr. ether on steam bath, dry at 100° to constant wt (ca 30 min.), cool, and weigh. Calc. % total fat on H_2O-free basis.

Weigh duplicate samples of 1 g (within ± 0.03 g) fat into 300 ml Florence flasks and add 4 ml glycerol-soda soln, 26.032(c). Heat flask carefully over asbestos gauze until bubbles start to appear, then hold flask ca 1" over the heated gauze until cloudiness or turbidity disappears and mixt. is perfectly clear. After mixt. first becomes clear, 30-60 sec. addnl gentle heating insures complete saponification. Cool; add few pieces of previously ignited pumice stone, 138 ml CO_2-free H_2O, and 3 ml H_2SO_4 (1+1); and proceed as in 26.033, using same app. Use 0.02*N* NaOH for titrn. Multiply ml 0.02*N* NaOH used by 1.1 and divide by wt fat used. Perform blank detn and make correction. Report number of ml 0.02*N* NaOH/g fat as "fat number."

Acetic and Propionic Acids (32)—
Official, Final Action

13.080 PREPARATION OF SAMPLE

(a) *Air-dried bread.*—Prep. sample as in 13.076, 13.077.

(b) *Fresh bread.*—For analysis of fresh product, which may be difficult to air-dry without slippage or loss of volatile acids, pass sample thru meat grinder equipped with ¼" hole plate, and divide finely by rubbing thru No. 8 sieve. Proceed with analysis promptly (24-48 hr) or preserve with $CHCl_3$ as follows:

To prepd bread in Mason jar filled to ¾ capacity, add washed $CHCl_3$ absorbed in ca 1 g cotton (ca 5 ml $CHCl_3$/pint container). Close jar tightly (self-sealing lids are recommended) and roll to mix contents thorely. Store samples at ca 25° or refrigerate where higher temps occur.

13.081 REAGENTS—*See* 18.017

13.082 APPARATUS

(a) *Distillation apparatus.*—Use steam distn app. 13.016(a), or gas-fired steam generator.

(b) *Chromatographic tubes.*—Approx. 15×250 mm or ca 15×450 mm, constricted at lower end to ca 4 mm i.d.

(c) *Test tubes.*—Approx. 16×150 mm, g-s.

(d) *Eyedropper pipet.*—Approx. 180 mm long.

13.083 DISTILLATION

Transfer 10 g of the air-dried bread or 15 g of the prepd fresh bread to 150 ml distg flask. Add 50 ml H_2O and 10 ml ca 1*N* H_2SO_4. Mix thorely and add 10 ml *20% phosphotungstic acid soln.* Mix by swirling and add 40 g $Na_2SO_4.7H_2O$. Swirl again to partially dissolve salt. Mixt. should now be acid to *congo red paper;* if not, acidify with H_2SO_4 (1+1). Connect to condenser and steam generator, heat contents of distg flask to boiling, and distill 200 ml in 35-40 min. (Connect steam source just before heating bread solids suspension to prevent clogging of steam tube and to agitate bread solids.) Keep vol. in distg flask at ca 60-80 ml by means of small burner.

Transfer distillate to 400–600 ml beaker, add ca 10 ml *ca 0.01N formic acid*, make alk. to phthln with ca 1N NaOH, and evap. to ca 5 ml. Transfer to 25–30 ml g-s. test tube, rinsing beaker with 3 portions H_2O. If insol. material adheres, add few drops of ca 1N H_2SO_4 with 1 rinse. Make alk. to phthln and evap. just to dryness by inserting tube in steam bath or in boiling H_2O. (Air jet hastens evapn.) Det. acetic and propionic acids in evapd distillate by the sepn technic, 18.023(d), after prepg, testing, and stdzg column as in 13.084.

13.084 CHROMATOGRAPHIC SEPARATION

(a) *Preparation of partition column.*—See 18.022. (Where amount of propionic acid approaches 20 mg in column and definite band is observed below propionic acid band, use the long chromatographic tube (450 mm) and ca 10 g silicic acid. Then take twice amounts of H_2O, indicator, and NH_4OH as used for 5 g silicic acid.)

(b) *Test of silicic acid for solubility and standardization of column.*—Prep. stock solns of formic, acetic, and propionic acids (reagent grade) by dilg 5 ml acid to 250 ml and stdze the acetic and propionic acids by titrg 1.0 ml aliquots with 0.01N NaOH, using cresol red indicator, to pink persisting ca 45 sec.

Prep. following dil. stock solns from the stock solns and boiled H_2O: Formic acid, 10 ml to 50 ml; acetic acid, 20 ml to 50 ml.

Prep. following trial mixts of formic acid and known amounts of acetic and propionic acids:

ACIDS AND WATER	MIXTURE A: STOCK SOLNS	MIXTURE B: STOCK SOLNS	MIXTURE C
	ml	*ml*	
Formic	10	10	1 ml dil. stock soln
Acetic	10	10	1 ml dil. stock soln
Propionic	10	30	1 ml stock soln
Water	20	None	None
Total vol.	50	50	3 ml
Test aliquot	1	1	3 ml

(Above mixts cover range of acetic and propionic acids usually present when 15 g fresh bread preserved with propionate is used as initial sample.)

Pipet indicated test aliquots from mixts *A* and *B* and entire mixt. *C* into bottom of g-s. test tubes (16×150 mm), neutralize with ca 1N NaOH, using phthln, and add 1 drop excess.

Proceed with evapn, sepn, and titrn as in 18.023(c) and (d). Calc. results for acetic and propionic acids to mg/100 g sample. Following factors are based on 15 g fresh bread or 10 g air-dried bread:

Fresh bread (mg/100 g):
Acetic acid = 4.00 × ml 0.01N $Ba(OH)_2$
Propionic acid = 4.63 × ml 0.01N $Ba(OH)_2$

Air-dried bread (mg/100 g):
Acetic acid = 6.00 × ml 0.01N $Ba(OH)_2$
Propionic acid = 7.40 × ml 0.01N $Ba(OH)_2$

(c) *Identification of acids.*—*See also* 18.024 and 13.087. With trial mixts *A*, *B*, and *C* and with most breads to which propionate has been added, only 3 definite bands appear on column and they elute in this order: propionic, acetic, and formic acids. Method provides for add'n of enough formic acid to supply definite following band to insure that acetic acid is completely eluted. Amount of mobile solvent required to move each acid from top of column to point of emergence is function of concn of that acid. For amounts of acetic acid normally present in bread, differences in threshold vol are not critical. To identify propionic acid by threshold vol., however, it is necessary to check threshold vol. for the concn indicated by titrn. Sepn of mixts *A*, *B*, and *C* supply enough data for amounts normally found. Threshold vols may be predicted for intervening concns by plotting concn against det'd threshold vols.

Due to differences in propionic threshold vol., make changes in mobile solvents from 1% butanol-$CHCl_3$ to 10% butanol-$CHCl_3$ at a constant vol. of 1% butanol-$CHCl_3$ rather than at propionic acid threshold, preferably when greater part of propionic acid has been eluted. Then acetic acid threshold vol. will not be affected by changes in propionic acid threshold vol.

Acids as sepd in butanol-$CHCl_3$ solns may be further identified by formation of mercurous acetate or mercurous propionate crystals (J. Assoc. Offic. Agr. Chemists 28, 644–645) or by paper chromatography, 13.087.

Identification of Volatile Acids by Paper Chromatography (a)—*Official, First Action.*

13.085 APPARATUS

Chromatographic tank and accessories.—See 24.108.

13.086 REAGENTS

(a) *Mobile solvent.*—Mix 2 vols acetone, 1 vol *tert*-butanol, 1 vol. *n*-butanol, and 1 vol. NH_4OH. Prep. fresh daily.

(b) *Chromogenic indicator.*—Add 200 mg of Methyl red bromcresol green to mixt of butanol, formalin and 10 ml alcohol. Adjust to pH 7.2 with 0.1N NaOH.

(c) *Standards.*—Pipet 1 ml each of acetic, propionic, butyric, and valeric acids into 1 ml vol. flasks and dil. to mark with 1.0 conc. stock soln. Pipet 1 ml each of [illegible]

sep. 25 [illegible] beaker; spot 1 ml each of the 4 stock solns into single 25 ml beaker to prep. known mixt. Neutralize acids and mixt. with 0.1 N NaOH, using cresol red indicator, 15.144(f), avoiding excess alkali. Evap. to ca 0.5 ml, or evap. just to dryness and take up in 0.5 ml H_2O.

13.087 TECHNIC

Steam distill 20 g well-mixed sample as in 13.083, collecting 200 ml distillate. Immediately neutralize distillate, using cresol red, 15.144(f), and 0.1 N NaOH. Evap. to 0.5 ml or evap. just to dryness and then take up in 0.5 ml H_2O. (To save time, place distillate in large porcelain evapg dish, and evap. to 5–10 ml; transfer to 25 ml beaker and evap. to 0.5 ml.)

With hard pencil, rule starting line 1" from bottom edge of 8×8" Whatman No. 1 unwashed chromatographic paper. Spot seven 1 µl spots with 1 µl pipet on paper, 1" apart, leaving 1" margin, to permit 1 spot for each acid, one spot for mixt., and two spots for unknowns. Spot in following order: acetic, propionic, unknown, mixt. of 4, unknown, butyric, valeric. Let dry. Clip paper to glass rod and suspend in tank with 50 ml mobile solvent in trough. (Since mobile solvent is heavy liquid, use 3 clips to fasten paper to glass rod to prevent sagging and to give more uniform solvent front. Do not sat. tank with mobile solvent before inserting paper.) Seal glass cover with cellophane or other suitable tape and let develop until solvent is ca 1" from top of paper. Remove paper from tank, let air-dry, and spray on front side with chromogenic reagent. (Spraying should be uniform and rather heavy but not to extent that chromogenic reagent runs or drips.) Faint yellow spots indicate presence of acids; heavier blue spots are due to Na ion. To intensify acid spots, place paper in atm. of NH_3 fumes momentarily (e.g., by placing ca 50 ml NH_4OH in 2 L beaker, rolling paper, and exposing to fumes by placing each end in beaker momentarily); entire paper immediately turns green. Remove paper from NH_3 fumes. Acids gradually appear as red spots, and presence of specific acids in sample may be detd by comparing their R_f values with those of mixt. and individual acids.

Since color of acids is not stable, mark spots with pencil as soon as they are completely developed.

13.088 ★ Citric Acid (%) — ★
Official, First Action

To wt air-dried bread equiv. to 77.7 g moisture-free bread, in 500 ml vol. flask, add 400 ml *alc. H_2SO_4-phosphotungstic acid soln* (25 ml 1 N H_2SO_4, 20 ml 20% phosphotungstic acid soln, and 55 ml H_2O, dild to 500 ml with alcohol). Shake 5 min., dil. to mark with the H_2SO_4 phosphotungstic acid soln, and let stand overnight. Readjust to mark with alcohol, shake 5 min., and filter with suction on paper in 12 cm büchner. Transfer 325 ml filtrate to centrifuge bottle, add 30 ml *Pb(OAc)₂ soln* (75 g of the salt + 1 ml HOAc dild to 250 ml with H_2O), shake 5 min., and centrifuge at ca 900 rpm 15 min. Decant supernatant (disregard turbidity), let drain, transfer residue with ca 150 ml H_2O to 250 ml vol. flask, and thoroly sat. with H_2S. Dil. to mark with H_2O, shake thoroly, and filter thru large folded paper. Proceed as in 20.051, using 200 ml aliquot.

13.089 Ash (35) — Official, Final Action

Use 3–5 g prepd sample, 13.076, and proceed as in 13.006 or 13.008.

13.090 Chlorides in Ash — Official, Final Action
— *See* 13.119

Iron — Official, Final Action

13.091 PREPARATION OF SAMPLE

Slice bread, let air-dry until in equilibrium with air, and crush to ca 20-mesh size on wooden surface with wooden rolling pin. (Grinding may be done in mill if experiments show no increase in Fe due to grinding of particular material under examination. In general, grinding in mills increases Fe content.)

Proceed as in 13.013.

13.092 Calcium — Official, Final Action —
See 13.014

Vitamins in Enriched Bread —
Official, Final Action

13.093 PREPARATION OF SAMPLE

Det. fresh wt of entire sample taken for drying, usually 6 loaves of 1 lb size or alternate slices from 6 loaves of 1.5 lb size. Slice unsliced bread into slices ca 1 oz each. Spread slices on coarse screens, elevated to provide good air circulation, and let air-dry until crisp enough for efficient grinding. (If riboflavin is also to be detd, drying should be done in absence of light.) Weigh air-dried bread and grind entire amount to pass No. 20 sieve. Mix well, and store in air-tight glass jars at ca 10°. Det. air-dry wt:fresh wt ratio for subsequent use in calcn of results to fresh basis.

Proceed as in 39.029–39.032, 39.033–39.036, and 39.037–39.039.

13.094 Protein — Official, Final Action

Det. N as in 2.044, using 2 g prepd air-dried ground sample, 13.076. Multiply % N by factor 5.7 to obtain % protein.

13.095 Fat (Acid Hydrolysis Method) —
Official, Final Action — *See* 13.019

and transfer to Mojonnier fat-extn tube. Rinse beaker and rod with 7 ml alcohol, add to extn tube, and mix. Rinse beaker and rod with 25 ml ether, added in 3 portions; add rinsings to extn tube, stopper tube with cork or stopper of synthetic rubber unaffected by usual fat solvents, and shake vigorously 1 min. Add 25 ml petr. ether (b. p. <60°) to extn tube and repeat vigorous shaking. Centrifuge Mojonnier flask 20 min. at ca 600 rpm and proceed as in **13.019**, beginning "Draw off as much as possible of ether-fat soln . . ."

Drying to constant wt takes ca 40 min. for fish. Long heating periods may increase wt of fat. If centrifuge is not available, extn can generally be made by letting Mojonnier flask stand until upper liquid is practically clear, then swirling flask and again letting stand until clear. If troublesome emulsion forms, pour off from Mojonnier flask as much of ether-fat soln as possible after letting flask stand, add 1-2 ml alcohol to Mojonnier flask, swirl, and again let mixt. sep.

Rapid Modified Babcock Method (10)—Official, First Action

(Applicable to raw, canned, and frozen fish)

18.014 DETERMINATION

Weigh 9.0 g ground and mixed sample into Paley-type Babcock cheese bottle (Kimble Glass No. 508, 20% size), stopper, and add ca 30 ml reagent prepd by mixing equal vols HOAc and 70-72% $HClO_4$. Place in H_2O bath (2 L stainless steel beaker is satisfactory) maintained at 92 ± 2°, swirling occasionally until no lumps remain (usually ca 20 min.). Remove from bath, add reagent until fat is well up in calibrated neck of bottle, centrifuge 2 min. at ca 600 rpm, and read % fat with dividers, using bottom of top meniscus. If fat falls below calibration, add more reagent, centrifuge 1 min., and read again.

With very fat fish, it may be necessary to use <9 g sample. In this case, correct reading by multiplying % fat by factor 9/g sample.

18.015 Fat in Fish Meal – Official, First Action

See **22.037**

Volatile Fatty Acids

Column Chromatographic Method (11)—Official, Final Action

18.016 APPARATUS

(a) *Steam distillation assembly.*—Fig. 18:1. Assembly consists of boiler flask (3 L) giving steam at constant rate so as to produce constant rate of distn, distn flask, condenser, and 200 ml vol. flasks as receivers. Std distn flask with side arm (ca 9 mm o. d.) attached near center of neck, and with steam inlet tube (ca 10 mm o. d.), is satisfactory. Heating coil of steam generator is made

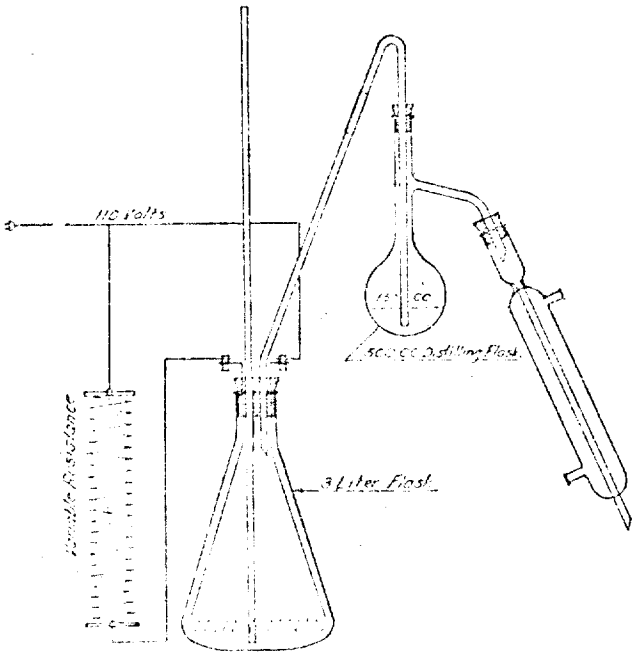

FIG. 18:1. STEAM DISTILLATION ASSEMBLY

by winding 5 feet 28 gauge Chromel wire (or equiv.) around hollow pipe ca 0.25" diam. and heating red hot to detemper wire. Leads into boiler flask are brass, Cu, or other non-ferrous metal ca 3/32" diam.

Any similar distn assembly may be used if it is of capacity to handle vols specified in method and gives 57 ± 2% recovery of acetic acid on distn.

(b) *Chromatographic tube.* Approx. 15×250 mm.

(c) *Source of air pressure or compressed N gas equipped with pressure regulator.*—If such source is not available, following system serves purpose: Fit 1 L side arm flask with 2 hole rubber stopper. Pass glass manometer tube 70 cm long thru one hole in stopper so that it reaches bottom of flask, and thru other hole pass glass tube ca 8 cm long, whose upper end is connected to top of chromatographic tube by rubber tubing. Connect rubber hand-aspirator bulb to side arm of flask. Fill flask with Hg to depth of 1.5 cm. (Height of Hg column in manometer tube indicates pressure in system; 25 cm is equiv. to ca 5 lb.) To maintain reservoir pressure when chromatographic tube is disconnected, fit stopcock into line leading from flask to chromatographic tube; and to prevent valve in hand aspirator from leaking, insert second stopcock between side arm and bulb.

(d) *Rubber bulb.*—5 ml. (Type used on dropping bottles.)

(e) *Micro funnel.*—Büchner type, 2 ml capacity with coarse fritted disk; Corning No. 36060.

18.017 REAGENTS

(a) *Butanol in chloroform.*—1%. Remove alcohol from USP $CHCl_3$ by washing 3 times with ½ vol. H_2O. Add 10 ml *n*-butanol to 1 L washed $CHCl_3$ in separator, shake vigorously, add 25 ml H_2O, and shake again. Let lower layer stand until clear, and drain. Discard aq. layer. Store in contact with granular anhyd. Na_2SO_4.

(b) *Butanol in chloroform.*—10%. To 900 ml USP $CHCl_3$ (*not* previously washed) in separator add 100 ml *n*-butanol, shake vigorously, add 25 ml H_2O, and shake again. Let $CHCl_3$ stand until clear, and drain. Discard aq. layer. Store in contact with granular anhyd. Na_2SO_4.

(c) *Alphamine Red R indicator.*—Dissolve 50 mg mono-NH_4 salt 3-(4-anilino-1-naphthylazo)-2,7-naphthalene disulfonic acid (Eastman No. 6410) in 25 ml H_2O. (Red soln produced by 1 drop indicator in 20 ml H_2O must be changed to violet by 1 drop 0.01*N* HCl.) Prep. fresh weekly.

(d) *Cresol red indicator.*—Dissolve 50 mg *o*-cresolsulfonphthalein in 20 ml alcohol, add 1.3 ml 0.1*N* NaOH, and dil. to 50 ml with H_2O. Use 2 drops for each 25 ml aq. soln.

(e) *Barium hydroxide soln.*—0.01*N*. (Store in polyethylene or paraffin-lined bottle and protect from CO_2 of atmosphere with soda-lime or Ascarite; dispense from 10 ml buret.) (0.01*N* NaOH may be used instead of $Ba(OH)_2$ except in titrn of Soln *B*, 18.020. NaOH soln must be used in titrn of Soln *A*, 18.020, since Na salts are required for chromatography.)

(f) *Sodium acetate-sodium chloride soln.*—Dissolve 12 g NaCl and 25 g $NaOAc.3H_2O$ in H_2O and dil. to 500 ml.

(g) *Silicic acid.* Reagent grade "100-mesh" powder, suitable for chromatography (Mallinckrodt Chemical Co. No. 2847 or equiv.).

18.018 STANDARDIZATION OF DISTILLATION APPARATUS

Place app., 18.016(a), in laboratory so that it is free from drafts and sudden changes in temp. Make mark on boiler flask at 1.5 L level, fill to this mark with H_2O, heat to boiling, and boil several min. before starting distn. Transfer 150 ml H_2O to distn flask, add 1 drop H_2SO_4 (1+1), connect condenser, insert steam inlet tube into distn flask, and bring contents of flask to incipient boiling with burner. Connect steam inlet tube with steam supply from boiler, and steam distill. Regulate rate of evolution of steam and height of small flame of burner under distn flask so that vol. of liquid in distn flask is kept constant at 150 ml and distillate collects at rate of 200 ml/hr. (Period of collection may vary 5 min. for 200 ml distillate. The 150 ml vol. in distn flask should remain constant within ±10 ml. Boiling may be stopped to permit test of constancy of 150 ml vol. by momentarily interrupting steam supply. Few trials will show conditions necessary to maintain constant vol. in distn flask and constant rate of distn.) Det. blank on 2 successive 200 ml portions of distillate by titrg with 0.01*N* alkali (phthln) in CO_2-free atmosphere.

Transfer 50 ml ca 0.1*N* HOAc (concn must be accurately known) to distn flask; add 1 drop H_2SO_4 (1+1) (avoid contact with neck of flask) and 100 ml H_2O. Collect 200 ml distillate and titr. with 0.1*N* alkali. Correct for titrn blanks and compute % acid distd. Distn technic and app. are satisfactory when recovery is 57 ± 2%. App. so adjusted gives recoveries (±2%) of formic, propionic, and butyric acids of 40.5, 81, and 92%, resp. (J. Assoc. Offic. Agr. Chemists 21, 684, 688 (1938)), on 200 ml distillate.

18.019 PREPARATION OF SOLUTION

Comminute sample (include entire contents of canned products) by passing 3 times thru food chopper, mixing after each grinding. Weigh 50 g comminuted material into tared 500 ml wide mouth erlenmeyer, add ca 150 ml H_2O, stopper flask, and shake vigorously [illegible]

thoro suspension of material. Add 25 ml 1N H_2SO_4, mix, ppt proteins with 20% *phosphotungstic acid soln* (40 ml is usually enough), make to 300 g with H_2O, shake vigorously ca 1 min., and filter thru 24 cm rapid folded paper.

18.020 DISTILLATION AND COMPUTATION OF VOLATILE ACID NUMBER

Pipet 150 ml prepd soln into distn flask of app. and make acid to *Congo red paper* with H_2SO_4 (1+4). Steam distill as in 18.018. Collect 200 ml distillate, titr. with 0.01N NaOH to phthln end point, and designate as *A*. Collect second 200 ml portion distillate, titr. with the 0.01N $Ba(OH)_2$ soln to phthln end point, and designate as *B*. To calc. volatile acid number multiply titrn obtained on distillate *A*, corrected for blank, by 4.

DETERMINATION OF INDIVIDUAL VOLATILE FATTY ACIDS

18.021 *Formic Acid (12).*

Add 2 drops satd $Ba(OH)_2$ soln to distillate *B*, 18.020, and evap. to dryness on steam bath. Add ca 5 ml H_2O to residue and 1 ml more of 1N HCl than necessary to liberate volatile acids. Filter thru small paper into 125 ml erlenmeyer with ⅌ joint, and wash paper with H_2O in such manner that total filtrate equals 30-40 ml. Add 10 ml of the NaOAc-NaCl soln and 10 ml *5% $HgCl_2$ soln*. Connect flask with ⅌ air condenser and place on steam bath 2.5 hr.

With suction thru glass siphon attached to funnel by rubber stopper, transfer ppt of Hg_2Cl_2 to previously weighed microfunnel, 18.016(c), provided with mat of asbestos ca 2 mm thick. Rinse flask with H_2O followed by alcohol. Dry 30 min. at 100°, cool, and weigh. Weigh funnel with another funnel, prepd with asbestos and treated similarly to one contg ppt, as counterpoise.

Wt Hg_2Cl_2 (mg) × 0.0975 = mg formic acid in distillate. To calc. total formic acid originally present in aliquot of sample in distn flask before distn, divide mg formic acid found by 0.24 (fraction formic acid distd in second 200 ml distillate) and multiply by 4 to obtain formic acid in 100 g sample being analyzed. (NOTE: Factor 4 applies only to products prepd as in 18.019. If other sample wts and aliquots are used as for eggs, 16.035, appropriate factor must be used.)

CHROMATOGRAPHIC SEPARATION OF C_2 TO C_6 SATURATED FATTY ACIDS (*B*)

18.022 *Preparation of Partition Column*

To 5 g silicic acid in glazed porcelain evapg dish add 1 ml of the Alphazoine Red R indicator soln and just enough 1N NH_4OH to give all color of the indicator (1 drop is usually enough). Add max. quantity of H_2O that the silicic acid will hold without becoming sticky or agglomerating in the butanol-$CHCl_3$ soln. (This quantity must be detd for each batch of silicic acid and usually varies from 50-75% of wt of silicic acid.) Mix thoroly with pestle until homogeneous. Add ca 25 ml 1% butanol in $CHCl_3$, and mix to form slurry that pours readily. Pour this slurry into chromatographic tube contg small cotton plug in neck of constricted end. To avoid air pockets tilt tube slightly while pouring. If air bubbles form while pouring, eliminate by stirring suspension in tube with long glass rod.

Clamp tube vertically in ring stand. In top insert 1 hole rubber stopper fitted with glass tube bent to 90° angle and held in place by Bunsen clamp against pressure to be exerted. Connect bent glass tube to pressure source, 18.016(c). Adjust pressure to 5-10 lb/sq. in., so that excess solvent is forced thru column dropwise.

During removal of excess solvent the gel packs down. As column packs down, particles of gel adhere to wall of the tube, but eventually gel leaves wall of tube relatively clean. This is point of optimum density for column, and column is ready for use. Apply pressure until solvent reaches surface of column. If solvent passes below surface, causing drying or "cracking" of column, or if air pockets are present, extrude packing from tube, reslurry with solvent, and repack column.

18.023 *Test of Silicic Acid for Suitability and Standardization of Column*

(a) *Preparation of standard acid solns.*—Pipet 1 ml of each of following acids into sep. 1 L vol. flasks: Acetic, propionic, butyric, and valeric. Dil. to vol. with H_2O, and mix. Pipet 10 ml of each soln into sep. 125 ml erlenmeyers and titr. with 0.01N NaOH, using cresol red indicator, to pink persisting ca 45 sec.

Ml 0.01N NaOH × normality × F = mg acid/ml std acid soln, where F = 6.01 for HOAc; 7.41, propionic; 8.81, butyric; and 10.2, valeric acid.

(b) *Preparation of standard acid mixture.*—Pipet 50 ml of each std acid soln into same 250 ml vol. flask and dil. to vol. with H_2O. Concn of each acid in std acid mixt. = 1/5 concn in std acid soln.

(c) *Preparation of known samples.*—Pipet 5 and 25 ml aliquots of std acid mixt. into sep. 50 ml beakers. Just neutralize with 0.1N NaOH, using phthln, and add 10 drops excess. Evap. to dryness on steam bath. If desired, chromatograph acids individually by using 5 ml aliquots of each std acid soln and 5 ml aliquots of 5 fold dilns of each std acid soln. Combinations approximating compositions in samples may also be prepd.

(d) *Column separation.*—(Good sepn and yields

depend upon transfer of sample to column with quantities of solvent specified.) To dry residue of Na salts add 2 ml of the 1% butanol in $CHCl_3$ soln and while stirring with glass rod add H_2SO_4 (1+1) dropwise until all Na salts are converted to free acids (acid to Congo red paper). Add 1 g anhyd. Na_2SO_4.

Acids elute in following order: Valeric, butyric, propionic, and acetic. Place 50 ml graduate under column as receiver. Decant supernatant onto column, pouring it slowly down side of tube without disturbing level surface of column. Apply pressure until solvent reaches surface of gel. Wash beaker with 1 ml solvent, pour onto column, and with stirring rod transfer residue in beaker to column. Apply pressure until solvent just disappears into Na_2SO_4 layer. Wash beaker with another 1 ml solvent, transfer to column, wash inside of tube with 1 ml solvent, and apply pressure until solvent just disappears into Na_2SO_4 layer. Fill tube with solvent and apply pressure. Each time front (lower edge) of a band reaches point 2-5 mm above narrowest portion of constriction of tube, record vol. collected and change receiver. For each acid, total cumulative vol. is threshold vol. used for identifying bands in succeeding runs. After propionic acid (third band) has been eluted, fill tube with 10% butanol in $CHCl_3$ and use this solvent to elute HOAc. (Propionic acid is eluted after ca 50 ml 1% butanol in $CHCl_3$ has passed thru column. Observe vol. actually used. In succeeding runs change to 10% butanol in $CHCl_3$ at that vol. whether or not propionic acid is present.)

Transfer eluates to sep. 125 ml erlenmeyers, rinsing each graduated cylinder with three 5 ml portions of H_2O. Add 1 drop of the cresol red indicator soln and titr. with 0.01*N* alkali. As end point approaches, stopper flask and shake vigorously to completely ext. acids from solvent phase. Correct titrn of each eluted band for blank as follows: Collect 25 ml of the butanol-$CHCl_3$ mixt. from column before any acids are transferred, add 15 ml boiled and cooled H_2O, and titr. as above with 0.01*N* alkali.

If bands are not clearly differentiated or recoveries are <90%, reject the silicic acid. (Additional stdzn with respect to threshold vol. may be desirable for identification in some instances, 18.024.)

18.024 *Identification and Determination*

Add 1 drop 1*N* NaOH to neutralized distillate A obtained in 18.020 and evap. to small vol. Transfer to 50 ml beaker, evap. to dryness on steam bath, and proceed exactly as in 18.023 d).

Identify acids by comparing their threshold vols with those for ex. same quantities and ratios of known acids found in stdzn procedure. Threshold vol. of given quantity of each fatty acid is characteristic and quite reproducible under similar conditions. However, if conditions change, such as by use of different batch of silicic acid or different quantity of same batch, or different quantity of H_2O, threshold vol. for each acid must be redetd. (If present, isobutyric acid is measured as *n*-butyric acid.) For further identification as characteristic salts see J. Assoc. Offic. Agr. Chemists 28, 644(1945). Acids may also be identified by paper chromatography, 13.087.

Mg acid/100 g sample = ml 0.01*N* NaOH (corrected for blank) × normality × F, where F includes equiv. wts of acids, corrected for distn recoveries, and dilns. F = 421 for HOAc; 366, propionic; and 383, butyric acid.

NOTE: Data is given apply only to products prepd as in 18.019. If other sample wts and aliquots are used as per 18.035, appropriate factors must be used.

Gas Chromatographic Method (A)—Official, First Action

18.025 REAGENTS

(a) *Acetone.*—Redistd.

(b) *Dichloroacetic acid (DCA) soln, ~0.5N.*—Redistill DCA, weigh 0.645 g into 10 ml vol. flask, and dil. to 10 ml with acetone. Prep. daily.

(c) *Methyl esterifier.*—Transfer 250 ml anhyd. MeOH to 500 ml flask, weigh, and cool in ice bath in hood. With flask in bath, add BF slowly thru glass tube into MeOH until 31.5 g BF is taken up. BF_3 must be passed thru glass tube before tube is placed in and until after it is removed from MeOH. If >31.5 g BF is added, dil. with MeOH until total ml MeOH is 8× wt BF in g. Weigh 7 g ethylhexanoic acid (capronic acid) into 225 ml erlenmeyer and add 70 ml 12.5% w/v BF_3-MeOH soln. Boil 3 min. on steam bath, cool, and transfer to 750 ml separator containing 30 ml H_2O and 140 ml petr. ether. Shake 3 min. Remove lower layer. Add 10 ml 5% Na_2CO_3 soln, stopper and shake. Remove lower layer. Add 5 ml H_2O to separator, shake, and discard lower layer once. Filter petr. ether layer thru [illegible] wool plug covered with Na_2SO_4. Evap. solvent to [illegible] solvent removed in N stream. [illegible] Methanolate, cool, [illegible] 17.2-17.5. Store in [illegible] refrigerator.

(d) [illegible] Methanolate [illegible] DCA [illegible] of acid soln.

f. *Standard acid soln.*—Formic, acetic, propionic, and butyric acids 0.05 N. Check purity of stock solns of acids normally by gas chromatography. Prep. solutions for calibration by pipetting calcd vol. of each acid into 200 ml vol. flasks (0.50 ml formic, 0.57 ml acetic, 0.75 ml propionic, and 0.92 ml butyric acid). Dil. to mark with acetone. Pipet 20 ml acid dilu into erlenmeyer, add 20 ml H_2O and 2 drops phthln, and det. acid content by titrg with 0.05 N NaOH.

Mg acid/ml std soln = ml 0.05 N NaOH × normality × F, where F = 2.30 for formic; 3.00, acetic; 3.70, propionic; and 4.40, butyric acid.

18.026 APPARATUS

Gas chromatographic equipment, column packings, and operating conditions other than those described below may be used if they provide at least equiv. peak sepn.

(a) *Gas chromatograph.*—Use gas chromatograph equipped with argon ionization detector and all-glass injection and column system.

Parameters for modified Research Specialties gas chromatograph:

Column temp. 100°, detector temp. 170°, injection zone temp. 200°, argon flow rate 50 ml/min, high voltage 1400 v, attenuation ×5, and 6' glass column.

Optimum conditions for gas chromatographic sepn are obtained when peaks for solvent, Me enanthate std, and formic acid are completely resolved. Std and acid peaks are sharp, although formic and acetic acid peaks are not completely resolved. Conditions vary to some degree from instrument to instrument and should be experimentally reestablished.

(b) *Preparation of column packing.*—Weigh 1.3 g ethylene glycol adipate (Applied Science Laboratories, State College, Pa.), into 150 ml beaker and dissolve by stirring in 70 ml acetone. Weigh 0.26 g 85% H_3PO_4 into second beaker and dissolve in 30 ml acetone. Combine both solns. Weigh 12.5 g Anakrom ABS (Analabs, P.O. Box 5215, Hamden, Conn.) into 500 ml round bottom flask, add coat'ng acetone soln, and evap. in rotating evaporator at reduced pressure in H_2O bath at 35° enough to prev. use of solvents.

Pack column evenly. Condition packed column overnight for >12 hr at 150° by passing argon or nitrogen thru column at ca 20 ml/min. Direct effluent thru 1/16" stainless steel tubing to small test tube shielded from heat of oven. Column is ready for use when bleeding of column substrate into test tube over 2 hr period has practically stopped.

18.027 CALIBRATION

Pipet, into one 5 ml vol. flask, vol. each std acid soln and vol. ME std as follows:

Individual Acid Concn in each, µg	C₁-C₄ Acid Std Soln 0.05 N, ml	ME Std	Vol. of Internal Std Added	
		ml	Concn	
< 1	0.25–0.5	1.0	dil. ME std	
1.0–2.5	0.5–1.0	0.5	ME std	
2.0–5.0	1.0–2.0	1.0	ME std	

Dil. to mark with acetone and mix. Repeat, using varying vols of acids to provide range of concn for each acid. Inject 3 µl aliquots into gas chromatograph. Measure peak heights on chromatograms and calculate R value for each acid: $R_A = h_A c_{ME}/c_A h_{ME}$, where c_{ME} and c_A = ng Me enanthate and acid, resp., in total vol. acetone soln, and h_{ME} and h_A = respective peak heights corresponding to the 3 µl injected.

When solvent peak tailing extends into region of ME and acid peaks, draw tail of solvent peak as it would normally appear if no other peaks were present. Use this curved extension as baseline for measuring heights of all peaks above it. Measure heights of remaining peaks to normal baseline.

18.028 DETERMINATION

Quantitatively neutralize steam distillate of volatile acids obtained by 18.020 with 0.01 N NaOH and phthln as indicator. Add 1 ml 0.1 N NaOH excess. If color is discharged, repeat addn. Conc. to ca 15 ml on steam bath in air stream. If color is discharged, add 0.5 ml more 0.1 N NaOH, transfer to 50 ml beaker, and evap. to dryness in air stream. Loosen salts with micro spatula and transfer carefully (thru funnel 1.5 cm diam.) into tube-type, gas. vol. flask (see following table, last column, for vol.).

0.01 N NaOH Conc Nearest Integer or Short Distillate Before Addn of 0.1 N NaOH, ml	Approx Total Conc. Gas Acetic Acid, µg	Vol. of Internal Std Added		Total Vol After Dilu ml
		ml	Concn	
1.0–1.5	0.6–0.9	1.0	dil. ME std	2.0
1.5–3.0	0.9–1.8	0.5	ME std	5.0
3.0–6.0	1.8–3.6	1.0	ME std	5.0
6.0–8.0	3.6–4.8	1.0	ME std	5.0
9.0–12.0	5.4–7.2	2.0	ME std	10.0

For fish samples, add 1 ml ME int. std; for egg samples, add only 0.5 ml, because volatile acids are more evenly distributed between formic and acetic acid.

Add to beaker, rinsing walls, vol. 0.5 N DCA, equiv. to total NaOH added to distillate. Add slight excess DCA and carefully observe liberation of volatile acids. Red due to phthln disappears before volatile acids are completely liberated. Add DCA until irregular, coarse particles of salts of volatile acids are completely replaced by fine dicrystal. Na dichloroacetate. Liberation of volatile acids can be accelerated by stirring

with thin (2-3 mm) glass rod. Transfer thru funnel into vol. flask. Repeat rinsing with 0.5 ml or more acetone, and add to vol. flask. Swirl flask to disperse salts and to liberate volatile acids. Pipet vol. of dil. ME std or ME std (as specified for acid content of sample in table above) to vol. flask and mix. Centrifuge 3 min. at low speed in g.s. tube to sep. ppt. Na dichloroacetate. Inject 3 µl clear soln in gas chromatograph. Measure peak heights as in 18.027 and calc. acid concns mg in total vol. acetone soln, with R values established in calibration. $c_A = k_A c_{ME}/R_A h_{ME}$.

Neither total vol. acetone soln nor injection vol is critical, as these values do not appear in expressions for calcg R values or concns of acids. Therefore use of internal std provides wide latitude in operating conditions. If heights of acid peaks on chromatogram differ considerably, first calc. concns represented by smaller peaks by reference to ME peak. Add known increments of internal std soln and develop addnl chromatograms for calcg higher concns of acids. If necessary, and particularly for off-scale peaks, also dil. test soln with acetone or operate electrometer or recorder at lower sensitivity settings.

Store acetone solns of acids and Me enanthate in refrigerator if they are to be chromatographed after day of prepn. If they are used after ca 5 days, det. new R values on std solns in acetone stored in refrigerator for same period of time.

To calc. mg of each acid in wt of sample used for distn, divide by fraction recovered in distn. Typical recoveries for first 200 ml distillate are: Formic acid 0.405, acetic acid 0.57, propionic acid 0.81, and butyric acid 0.92 (*see* 18.018, last par.).

Histamine-Like Substances

Biological Method (15)—Official, Final Action

18.029 APPARATUS

(a) *Kymograph.*—Kymograph with horizontal muscle lever arm having friction or gravity writing point.

(b) *Muscle bath.*—At least 50 ml capacity, surrounded by 37° constant temp. bath. *See* Fig. 18:2. May be conveniently filled and emptied thru 3-way stopcock; 1 tube connected to reservoir of Ringer-Locke soln thru bulb or coil immersed in bath; other tube connected to vac. thru suction flask as waste receiver. Bubble air, filtered thru cotton, slowly and continuously around intestine from fine capillary tube.

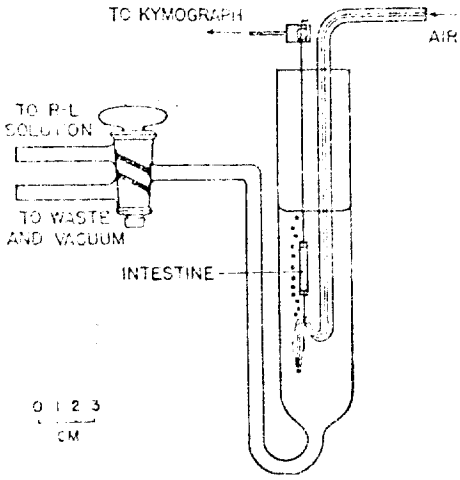

FIG. 18:2.—MUSCLE BATH

18.030 REAGENTS

(a) *Sodium chloride stock soln.*—180 g/L.

(b) *Potassium chloride stock soln.*—12 g/500 ml.

(c) *Sodium bicarbonate stock soln.*—15 g/500 ml.

(d) *Atropine sulfate stock soln.*—1.0 g/500 ml.

(e) *Calcium chloride stock soln.*—24 g anhyd. salt/500 ml.

(f) *Ringer-Locke soln.*—NaCl 0.9%; KCl, 0.042%; $CaCl_2$, 0.021%; NaHCO[illegible] [illegible] dextrose, 0.1%; and atropine sulf[illegible] Add 100 ml (a) and 10 ml (b) [illegible] 2 L vol. flask. Add H_2O to vol. of ca 1800 ml and then 10 ml (c) while swirling. Add 2 g anhyd. dextrose before use. Dil. to vol. with H_2O. Keep soln contg dextrose in refrigerator when not in use, discarding when it becomes moldy.

(g) *Histamine diphosphate std water soln.*—0.1 mg histamine diphosphate/ml in boiled H_2O. If stored in refrigerator when not in use, it will keep at least 3 months. Prep. dilu stds of 0.01 mg/ml and 0.005 mg/ml as required. If bath smaller than 50 ml is used, prep. dilns of (g) with (f) to avoid dilu of bath when stds are added.

(h) *Guinea pig intestine.*—Use guinea pigs weighing 300-400 g. Starve pig 24 hr, kill by blow on head, and remove intestine, severing at point proximal to ileocecal junction, retaining ca 12 cm of terminal ileum. Wash this section with soln (f) and use ca first 2 cm for first series of assays. Place remainder of intestine on cork in petri dish, just covering with soln (f). Prop fill[illegible] air and store in refrigerator at [illegible] 15 hr. Below 10 hr intestine loses its [illegible] Use addnl portions of intestine as required as long as material shows enough response to be [illegible] stimuli. Usually [illegible] hrs. Distal and proximal parts of intestine are not as sensitive [illegible] uniform response with barium not [illegible] [illegible] recovered after storage.

test paper no longer shows color (indicating complete adsorption); then filter thru dry paper. Save filtrate for detn of NaCl and Na_2SO_4.

35.108 DETERMINATION

To 50 ml aliquot filtrate in 250 ml g-s. flask add 2 ml ca 6N HNO_3, 10 ml of the $AgNO_3$ soln (more if quantity of Cl is large), and ca 5 ml *nitrobenzene*. Shake flask vigorously until AgCl coagulates, add 1 ml of the Fe alum indicator, and titr. excess $AgNO_3$ with the NH_4CNS soln. Take as end point first definite color that persists after shaking 1 min. Calc. NaCl from net $AgNO_3$ titrn on basis of 195 ml total vol., since 10 g C occupies 5 ml.

Net ml $AgNO_3 \times$ NaCl equiv. $\times 195 = \%$ NaCl.

Sodium Chloride in Basic Dyes (24)

35.109 PREPARATION OF SOLUTION

Dissolve 2 g sample in exactly 200 ml H_2O. Add 10 g activated C, 35.106(d), stir 1 min., and test for complete adsorption as in 35.107. Add C in 2 g portions, with stirring, until test paper shows no color; then filter thru dry paper.

35.110 DETERMINATION

Evap. 50 ml aliquot filtrate to dryness, and heat to volatilize any NH_4Cl. Transfer residue to 250 ml flask with ca 50 ml H_2O, and det. NaCl as in 35.108. Net ml $AgNO_3 \times$ NaCl equiv. $\times 200 = \%$ NaCl.

Sodium Sulfate (24)

35.111 REAGENTS

(a) *Barium chloride soln.*—1 ml = ca 0.0025 g Na_2SO_4. Stdze gravimetrically.

(b) *Tetrahydroxyquinone indicator.*—See 35.026(c). (In neutral soln contg equal vols alcohol and H_2O, indicator changes from yellow to red when Ba ions are present, and end point is observed by transmitted light. After some practice, results can be reproduced to ±0.05 ml of the $BaCl_2$ soln. Blank of ca 0.1 ml should be deducted.)

(c) *Phenolphthalein indicator.*—0.5% soln in 50% alcohol.

35.112 DETERMINATION

Acid or basic dyes.—Place 25 ml filtrate, 35.107 or 35.109, in 125 ml erlenmeyer, add 1 drop of the phthln, and make alk. by dropwise addn of ca 0.05N NaOH; then add ca 0.002N HCl dropwise until indicator is decolorized. Add 25 ml alcohol and ca 0.2 g of the tetrahydroxyquinone indicator. Titr. slowly with the $BaCl_2$ soln, shaking constantly, to red end point. Det. blank on reagents. From net titrn calc. as follows: Acid dyes: Net ml $BaCl_2 \times Na_2SO_4$ equiv. $\times 400 = \%$ Na_2SO_4; Basic dyes: Net ml $BaCl_2 \times Na_2SO_4$ equiv. $\times 400 = \%$ Na_2SO_4.

35.113 Sodium Halides in Halogenated Fluorescein Colors

Place 5 g sample in 400 ml beaker and add ca 150 ml H_2O. (If sample is a color acid, add just enough 10% NaOH soln to give complete soln.) Heat soln nearly to boiling and add 5 ml H_3PO_4. Digest soln until ppt of color acid is well coagulated, cool to room temp., transfer to 250 ml vol. flask, and dil. to vol. Mix thoroly, filter thru dry fluted paper, and discard first few ml filtrate.

(a) *Sodium iodide.*—Transfer 100 ml aliquot filtrate to 500 ml tall beaker; add 2.5 ml 30% NaOH soln, few glass beads, and 15 ml satd $KMnO_4$ soln, 35.096(a). Proceed as in 35.097, beginning "boil 5 min. . . ."

1 ml 0.1N $Na_2S_2O_3$ = 0.002498 g NaI.

(b) *Sodium bromide.*—Place 100 ml aliquot filtrate in 500 ml I flask and proceed as in 35.099, beginning "Add ca 12 ml H_3PO_4 . . ."

1 ml 0.05N $Na_2S_2O_3$ = 0.00257 g NaBr.

(c) *Sodium chloride.*—Place 100 ml aliquot filtrate in 400 ml beaker, heat to boiling, and add enough 10% $AgNO_3$ soln to ppt halides. Digest soln until ppt is well coagulated, cool, and transfer ppt to weighed gooch. Wash ppt thoroly with H_2O and alcohol. Dry crucible and contents at 135°, cool, and weigh.

If sample contains no NaBr or NaI, NaCl content can be calcd directly from wt ppt. If other halides are present, deduct wt Ag halide equiv. to I or Br found, (a) or (b), from wt ppt before calcg NaCl content.

AgCl × 0.4078 = NaCl; NaI × 1.5662 = AgI; and NaBr × 1.8248 = AgBr.

Sodium Acetate

35.114 APPARATUS

App., Fig. 35.5, can be assembled from stock items. Dist. flask is 125 ml acetylation flask with ₸ 24/40 joint. Receiver is 300 ml flask.

35.115 REAGENTS

(a) *p-Toluenesulfonic acid.*—Dry [illegible] toluenesulfonic acid monohydrate overnight at 110°, cool, and grind to powder.

(b) *Silver toluenesulfonate.*—Dissolve Ag[illegible] or Ag_2CO_3 in ca 10% excess of [illegible] soln, evap. to dryness, and dry 8 hr at 135°.

(c) *m-Cresol purple indicator.*—Triturate 0.5 g m-cresolsulfonphthalein with [illegible] 1N NaOH and dil. with H_2O to 100 ml.

35.116 DETERMINATION

To 500 ml erlenmeyer add 100 ml H_2O, 1 drop of the m-cresol purple indicator, and enough 0.1N

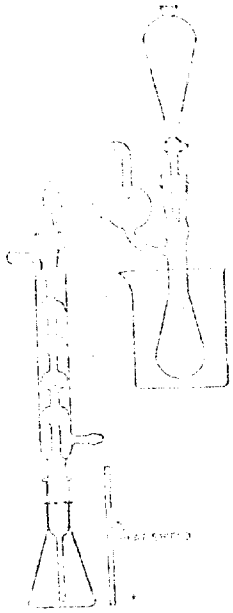

FIG. 35.5.—SODIUM ACETATE APPARATUS

NaOH or 0.1N HCl to turn color of soln just yellow; place flask under condenser.

Transfer 50 ml absolute alcohol to distn flask and add thru powder funnel 5.00 g sample, 5 g of the p-toluenesulfonic acid, and 1 g of the Ag toluenesulfonate. Add 3-4 pieces Alundum or other antibumping agent and mark level of liquid in distn flask. Wash funnel and neck of flask with 25 ml absolute alcohol. Shake flask to mix contents thoroly and attach it to condenser.

Immerse distn flask as far as possible in beaker of hot H_2O and heat H_2O to boiling. After ca 25 ml distillate collects, remove heat source and slowly add 25 ml absolute alcohol to distn flask. When contents of flask again begin to distill gently, replace heat source and collect second 25 ml distillate. Make third addn of alcohol and distill in similar manner. Finally boil until distn rate is slow (ca 30 min. for total time from beginning of first distn).

Wash 50 ml H_2O down condenser into receiver, and add 50.0 ml 0.1N NaOH. Add 3-4 pieces Alundum or other anti-bumping agent and connect to reflux condenser fitted with absorption tube contg Ascarite or other CO_2-absorbing material. Reflux 10 min., cool to room temp., add few drops of the *m*-cresol purple indicator, and titr. with 0.1N HCl to yellow-green that does not change in hue on further addn of acid. Det. blank by repeating procedure, omitting sample.

Calc. NaOAc from net vol. std NaOH soln required. 1 ml 0.1N NaOH = 0.0082 g $C_2H_3O_2Na$.

SOLUBLE MATTER—OFFICIAL, FIRST ACTION

35.117 Water-Soluble Matter

Place 5 g well-pwd. sample in 500 ml erlenmeyer or wide-mouth bottle, add 200 ml H_2O, stopper, and shake vigorously. Repeat mixing several times during 2 hr period. Filter mixt. and evap. 100 ml filtrate in weighed Pt dish on steam bath. Dry in oven at 100-105°, cool in desiccator, and weigh. Report increase in wt as H_2O-sol. matter. Test small portions of remainder of filtrate for chlorides, sulfates, and nitrates. If more than traces are present, make proper analyses on aliquot portions of filtrate.

35.118 Chloroform-Soluble Matter

Weigh 5 g sample into cellulose thimble and ext. in Soxhlet app. with $CHCl_3$ 16 hr. Transfer ext. to separator and wash with 30 ml portions H_2O until washings are practically colorless. Ext. combined washings with ca 30 ml $CHCl_3$ and add washings to main ext. Drain $CHCl_3$ ext. into weighed dish. Wash separator with few ml $CHCl_3$ and add washings to dish. Evap. at room temp. and dry in desiccator to constant wt (±0.5 mg), weigh, and report as $CHCl_3$ ext.

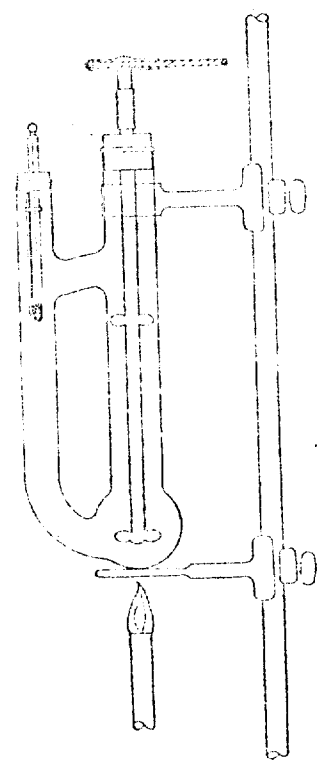

FIG. 35.6.—MELTING POINT APPARATUS

Proc. Soc. Exp. Biol. Med. 37: 131-134, 1937

9480

Effect of Various Salts on Hemolysis and Skin Necrosis Produced by Staphylococcus Toxin.

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Volk and Lipschutz[1] apparently were the first to study the action of hypertonic salt solutions on staphylococcus hemotoxin. The salts used were NaCl, Na_2SO_4, $MgSO_4(NH_4)_2$, SO_4 and $BaCl_2$. It was their opinion that staphylococcus toxin was not destroyed but that the hemolytic action was inhibited in the presence of hypertonic concentrations of the neutral salts with one exception and that was with $BaCl_2$.

Recently one of us has studied the effect of NaCl on the hemolytic and skin necrotizing factors in staphylococcus toxin.[2,3] It was found that a hypertonic solution of NaCl inhibited the degree and rate of lysis of rabbits' red blood cells. When a 5% concentration of NaCl was injected intradermally in rabbits prior to the intradermal injection of staphylococcus toxin skin necrosis was inhibited at the site of toxin injection.

The above observations suggested a further study of the effect of

[1] Volk, R., and Lipschutz, B., *Wien. Klin. Wchnschr.*, 1903, **16**, 1394.
[2] Rigdon, R. R., *J. Infect. Dis.*, 1937, **60**, 25.
[3] Rigdon, R. H., *Arch. Path.*, 1936, **22**, 763.

TABLE I.

Effect of Various Salts on Hemolysis Produced by Staphylococcus Toxin.*

Salt	Hemolysis: Salt + R.B.C.			Hemolysis: Salt + R.B.C. and Staphylococcus Toxin		
Concentration	M/1	M/2	M/4	M/1	M/2	M/4
NH_4Cl	4	3	—	4	4	—
NH_4Br	4	2	—	4	4	—
NH_4F	0	2	4	0	3	4
NH_4NO_3	4	3	—	4	4	—
$(NH_4)_2SO_4$	±	0	3	3	4	4
NH_4CNS	4	4	4	—	—	—
$(NH_4)_2HPO_4$	0	3	2	4	4	4
$(NH_4)_3Cit$	P	P	P	—	—	—
$(NH_4)_2Ox$	4	4	4	—	—	—
$NH_4Lact.$	4	4	4	—	—	—
$NH_4Acet.$	4	4	4	—	—	—
LiCl	0	0	0	3	4	4
LiBr	1	0	0	2	4	4
$LiNO_3$	±	0	0	±	3	4
Li_2SO_4	0	0	0	±	3	3
Li_3Cit	±	±	±	±	0	1
Li_2Ox	0	0	1	2	4	4
LiLact	0	0	0	1	1	4
LiAcet	0	0	0	1	3	3
NaCl	0	0	0	1	4	4
NaI	1	0	0	0	3	4
NaBr	0	0	0	0	3	4
NaF	4	4	4	—	—	—
$NaNO_3$	0	0	0	0	3	4
Na_2SO_4	0	0	0	3	3	4
NaCNS	4	0	0	1	2	4
Na_2HPO_4	4	4	4	—	—	—
Na_3Cit	±	±	±	4	4	4
Na_2Ox	i	i	±	i	i	4
NaLact	0	1	0	4	3	4
NaAcet	0	0	0	3	3	3
KCl	0	0	0	3	4	4
KI	5	0	0	0	2	4
KBr	0	0	0	0	4	4
KF	1	±	0	3	4	4
KNO_3	0	0	0	0	3	4
K_2SO_4	i	0	0	i	3	4
KCNS	4	3	1	4	4	4
K_2HPO_4	0	0	0	3	3	4
K_3Cit	±	±	0	4	4	3
K_2Ox	0	0	0	3	3	4
KLact	0	0	0	3	3	4
KAcet	4	1	1	—	4	4
$MgCl_2$	0	0	6	0	1	4
$MgBr_2$	1	4	3	4	4	3
$Mg(NO_3)_2$	3	±	0	1	0	±
$MgSO_4$	±	0	0	±	1	2
$Mg(Acet)_2$	1	1	1	1	2	3
$CaCl_2$	2	2	1	2	2	2
$Ca(NO_3)_2$	2	1	±	P	P	P
$SrCl_2$	3	±	0	2	1	2
$Sr(NO_3)_2$	P	2	±	P	P	P
$BaCl_2$	±	0	0	±	±	1
$Ba(NO_3)_2$	i	i	0	i	i	1

*1, 2, 3, and 4, degree of lysis.
P, heavy precipitate.
i, insoluble.
—, not done.

various salts on the hemolytic and skin necrotizing factors in staphylococcus toxin. The salts studied are given in Table I. The toxin preparation is the same as previously used.[2, 3] Washed rabbits' red cells were added to M/1, M/2, and M/4 solutions of the different salts to make a 1% suspension in a 2 cc. volume. The least amount of toxin which would completely hemolyse this concentration of cells suspended in 2.0 cc. of physiological saline was the amount of toxin used in all the tests. The toxin was added not less than 10 minutes after the cells were placed in the salt solutions. The titrations were read after standing at room temperature over night. The control titrations were run without toxin. The pH of all the salt solutions was determined.

Table I shows the effect on the blood cells of the various salts alone and in the presence of the hemolyzing unit of staphylococcus toxin.

From this table it can be seen that certain salts are hemolytic, while other salts are either slightly hemolytic or entirely non-hemolytic. Furthermore NH_4F, LiBr, $LiNO_3$, Li_2SO_4, Li_3 citrate, Li lactate, Li acetate, NaCl, NaI, NaBr, $NaNO_3$, NaCNS, Na acetate, KI, KBr, KNO_3, inhibit the hemolytic action of staphylococcus toxin. Of these salts the halogens and the nitrates show the strongest inhibiting effect.

The methods used to study skin necrosis are the same as given previously.[3] All the salts used in the hemolytic study were injected into rabbits intradermally in 0.5 to 2.0 cc. amounts.

A majority of these salts produced some skin necrosis in either M/1, M/2 or M/4 concentration. Solutions of NH_4Cl, LiCl, NaCl, K_2SO_4, Na lactate, Na acetate, and $MgSO_4$ failed to produce necrosis in the skin in the concentrations used. When either LiCl or $MgSO_4$ in 2 cc. quantities was injected intradermally immediately to 15 minutes before the injection of toxin no skin necrosis occurred at the immediate site of toxin injection. An equal volume of K_2Ox similarly injected into rabbits increased the extent of the skin necrosis.

Several of the results of the above experiments are particularly interesting. First, the effect of certain salts on the hemolysin and the skin necrotizing factors in staphylococcus toxin should be noted. This is strikingly illustrated by LiCl and $MgSO_4$. The former salt inhibits necrosis at the site of injection but has little or no effect on inhibiting hemolysis. The latter, $MgSO_4$, shows a marked suppression of both the hemolytic and skin necrotizing factors. Secondly, the effect of certain salts alone on rabbits' red blood cells and skin

Effects of Intravenous versus Intraruminal Injections of Acetate on Feed Intake of Goats

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Abstract

Sodium acetate injections into the rumen depressed feed intake while the same amounts injected intravenously had no such effect. Specifically, goats fed a grain ration ad libitum were injected either intraruminally or intravenously with acetate solutions concomitantly with eating spontaneous meals. Five-minute injections of acetate given intravenously caused blood levels to increase to 8 to 12 mM while injections given intraruminally caused an increase of only 0.5 to 1.0 mM. These data suggest that receptors sensitive to acetate are more likely to be situated in the lumen side of the ruminoreticulum than in an area where they would respond to blood levels. Although it seems apparent that there are acetate receptors in ruminants, it cannot be concluded that the feed intake depression following an acetate injection is related to satiety.

The volatile fatty acids, acetic acid in particular, have been suggested as a possible feedback component initiating satiety in ruminants. A severe depression of feed intake in ruminants in one study occurred during an eight-hour intravenous (jugular) injection of acetic and propionic acids and their sodium salts (11). The rate of injection was sufficient to provide in eight hours 12.5% of the estimated digestible energy maintenance requirement for 24 hours for the dairy cows. In another study with sheep trained to eat a single two-hour meal per day, jugular vein injections of sodium acetate at approximately 60% of the rate of the above study per unit metabolic mass failed to cause a feed intake depression (13). The injection was initiated about two hours before feeding. The rate was such that at the time of feeding the blood acetate concentration had been increased to a level comparable to maximum postprandial values.

Intraruminal injections of acetic acid and sodium acetate in cattle (18, 19), sheep (6, 20), and goats (4) cause depression of feed intake. It has been suggested that this depression is the result of a change in blood acetate concentration, which acts on hypothalamic receptors (9, 11, 15, 19, 20).

We previously perfused the cerebral ventricular system of hungry goats with 50 mM sodium acetate solutions (approximately [illegible] times the level in blood) and found no depression of feed intake (2). This was taken as evidence that the ventromedial areas of the hypothalamus, which are adjacent to the third ventricle, do not contain acetate receptors. Baile and Pfander previously suggested that there may be acetate receptors in the rumen (6).

This study was designed to test for the effects on feed intake of intravenous versus intraruminal acetate injections made only during eating by goats of voluntary meals. We studied the goats under ad libitum eating conditions, so that voluntary meal initiation and termination signals were not as likely to be suppressed by conditioned eating as under controlled feeding schedules. This is probably the reason why it has been our experience that acetate depression of feed intake is most easily obtained when injections are given during spontaneous meals, rather than continuously or during controlled interval meals.

Experimental Procedures

Four goats, each with a rumen fistula fitted with a cannula, were used in this experiment. The quantities of grain ration eaten (<16% fiber) (Omolene X, Ralston Purina) and water drunk were measured each day. A grain ration was selected since an energy balance regulation independent of bulk factors is best demonstrated on grain rations, and this may indicate that a chemo-feedback mechanism is more highly sensitized. The head of each goat was locked in a stanchion of a 0.7- by 1.4-m elevated pen in a room which was maintained at [illegible] and kept lighted 24 hours a day. For these treatments involving intravenous injections, polyethylene catheters were put in a jugular vein. The intraruminal injections were made via the ruminal cannula.

First the effects of intravenous injections were tested. When a relatively high acetate concentration was reached, the site of injection was

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changed to intraruminal. Three of the goats were tested several times with the highest level of acetate, and one test of an intraruminal injection was given immediately following a period when no injection was given. The injections were given only while a goat was eating. The duration of a treatment was two or three days to test for immediate and short-term effects. Our starting point for a rate of injection was in the order of 2 to 4 mmoles per minute which is similar to the normal uptake rate from the rumen. The concentration of the solution injected was increased geometrically starting with either 0.5 or 1.0 M acetate solutions. The intravenous injections were given, except in the case of one animal, at the rate of 4 ml per minute and were 0.9% NaCl, 0.5 M acetate (Fisher NF), 1.0 M acetate, 2.0 M acetate, 1.0 M acetate, and in several tests on one goat, 8 ml per minute of 4.0 M acetate. We tested only the higher concentrations of acetate solution intraruminally. All acetate solutions were adjusted to pH 7.34. The actual order, solutions tested, and quantity of acetate injected varied from animal to animal and are shown in the figures.

To simulate the effect on blood acetate levels of a short meal during these treatments, a series of injections at the rate of 4 ml per minute of acetate solution were made, both intravenously and intraruminally, for a period of five minutes in the goats. Serial blood samples were taken from a polyethylene catheter directed toward the head in a member vein to measure the rate and magnitude of change and the duration of the elevated metabolite level. The injections were made via a pump (Model [illegible], Harvard Apparatus) through a polyethylene catheter into the rumen or into a jugular vein (toward the heart). Ten ml blood samples were collected in syringes, transferred to test tubes which contained heparin and NaF, and used for acetate (C) and glucose (Technicon Autoanalyzer micro glucose method) (E7).

The following is a description of the equipment used. In front of the stanchion of each goat a 1.1 by 30.5 by 45 cm plywood board with two 21.5 cm diameter holes, 9 cm apart, held centered about 23 cm above and parallel to the floor of the goat pen. Attached 42 cm below this plywood board was another plywood board with two 15 cm diameter holes which were aligned with the holes of the top board. Between these two boards a 7 watt 3 ft. was placed between the holes approximately from the light, a photocell which typically 3 amp, 28 VDC (Model VAC) was placed on the edge of each hole in such a manner that, when a goat inserted its head in a hole to either drink or eat, the light beam impinging on the photocell would be broken. Below this assembly, a shelf held buckets for either water or feed. Four stanchions were used in these studies.

The relay switches of the photocell activated the relays, mounted on a rack in such a way that a counter (E3700A Grason-Stadler) and an event chart recorder (92 Rustrak Instruments) recorded each time a goat ate or drank. When a goat ate, a timer was activated (Haydon time delay timers, series DA-41) so that if the goat held its head in the eating position for as long as three seconds, a peristaltic pump (Model 090-1200, Harvard Apparatus) was activated as well as a three-way Solenoid Valve (no. 8314 A 41 Apparatus Service Co.). The valve, when activated, directed the solution being pumped to the goat via a polyethylene tube. Only one pump was used for the four animals; thus any goat could cause the pump to be activated, but only the goat or goats eating would receive an injection. Another timer (Automatic Feed Dial Timer, Automatic Timing and Controls, Inc.) in the circuit with the relays prevented any one single injection from being longer than 30 minutes. This prevented an overdose in the event of a light bulb going off, food blocking the light beam impinging on the photocell, etc. The solution to be injected was contained in a 1,000 ml graduate. The rate of injection per tube was constant in all experiments at about 4 ml per minute. To keep the intravenous catheters open, we injected about 0.05 ml saline every five minutes with a syringe pump (100-910 Harvard Apparatus). The pump was controlled by a timer (Industrial Timer Corp. Series TDAF or M) and was activated every five minutes for a duration of five seconds, controlled by another timer. We further incorporated a one way valve (B-D no. 3110 V) in series with the tubing between the solenoid valve and the animal to avoid any back flow of blood.

Results

Figure 1 shows typical changes in the blood acetate during and following intravenous and intraruminal acetate injections of five minutes. Clearly the intravenous injections caused a much greater increase in the blood acetate level than the intraruminal injections. The blood glucose levels were unaffected.

The feed and water intakes and the doses injected are shown in Figures 2 through 5. The figures show both the milliliters of solution and the equivalent of Na+ injected; where indi-

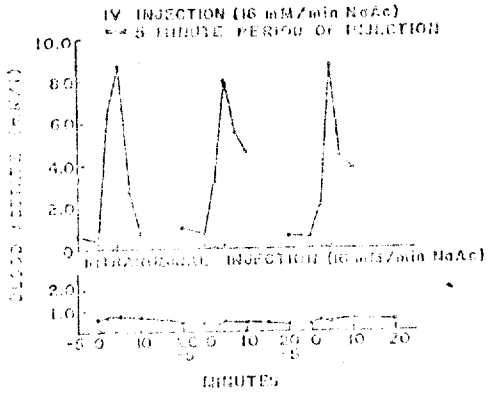

FIG. 1. Typical blood acetate concentration changes caused by an intravenous versus an intraruminal injection of sodium acetate.

um acetate was given, the equivalent of Na+ is equal to the moles of acetate. The goats occasionally reduced their feed intake during the intravenous acetate treatments which resulted in higher blood acetate concentration, but invariably reduced their intake much more during intraruminal acetate injections. Figures 2 through 5 show that in most cases the goats received less sodium acetate during the intraruminal injection treatments than during the intravenous injection experiments. The water intake invariably increased during the treatments involving sodium acetate injection. Quite often the intravenous sodium acetate injections

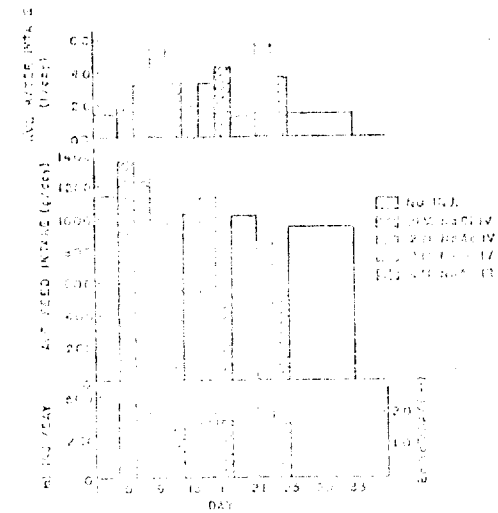

FIG. 2. The grain and water intakes and the equivalents Na+ and milliliters of solution injected either intravenously or intraruminally per day for the treatments of Goat 1.

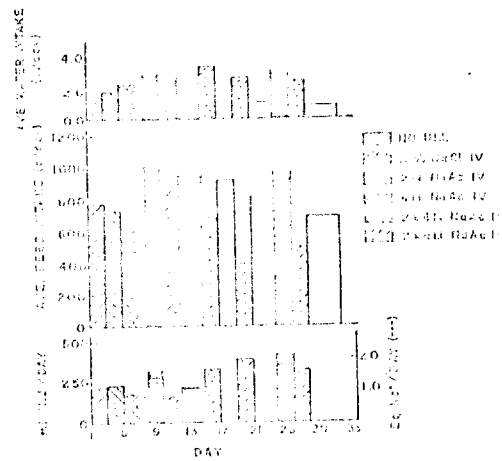

FIG. 3. The grain and water intakes and the equivalents of Na+ and milliliters of solution injected either intravenously or intraruminally per day for the treatments of Goat 2.

caused a larger increase of water consumption than did intraruminal injections.

The data in Figure 3 are from a hypothalamic hyperphagic goat [as previously described (5)] in the static phase. This animal weighed about 75 kg, while the other animals weighed about 25 kg. It is interesting to note that this animal responded in the same way as did the neurologically intact animals. Since

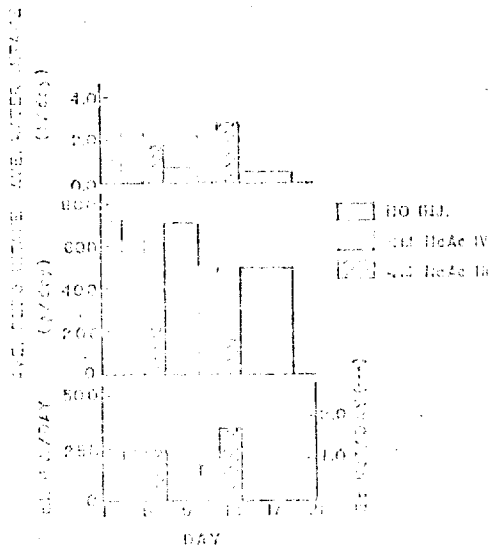

FIG. 4. The grain and water intakes and the equivalents of Na+ and milliliters of solution injected either intravenously or intraruminally per day for the treatments of Goat 3.

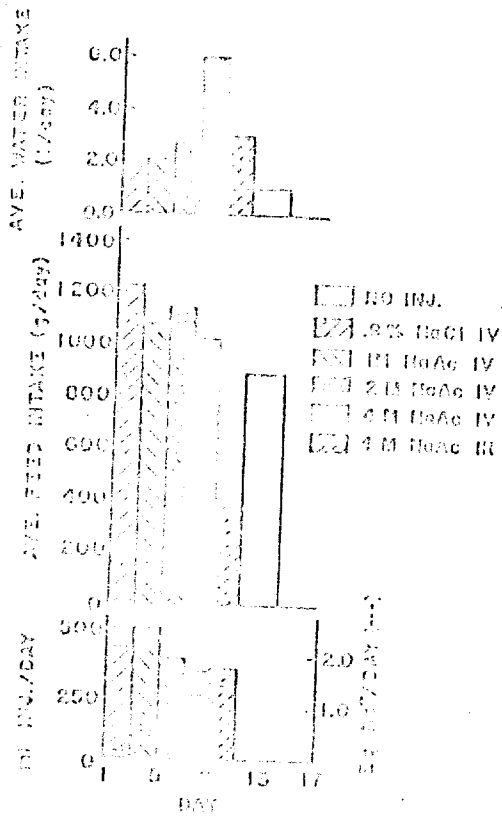

FIG. 5. The grain and water intakes and the equivalents of Na^+ and milliliters of solution injected either intravenously or intraruminally per day for the treatments of Goat 4.

this animal was so large, a 32 mmole per minute dose rate was used, the highest in these experiments.

We could detect no trend of a change in meal pattern during the various treatments. The animals normally ate 5- to 10-minute meals.

Discussion

These experiments clearly show that a sodium acetate solution injected intraruminally while a goat is eating is much more effective in depressing feed intake than the same solution injected intravenously under the same conditions. Our experiments further show that even when the jugular blood acetate concentration is increased during the course of a 5-minute meal, to four or five times the concentration found postprandially in the portal vein (8 to 12 mM versus 1.3 (1); or 2.5 mM (5)) or at least two times that of the ruminal vein (10, 17), the feed intake of goats is not appreciably depressed. These same injections in the rumen cause jugular blood acetate changes only one-tenth those after intravenous injections. This indicates that receptors for acetate, the activation of which results in the depression of feed intake, are not in the hypothalamus or in the ruminal or portal venous system. Our data indicate that the proposed receptors reside in the gastric area on the lumen side.

Our experiment differs from that of Dowden and Jacobson (11), first in that ours was with goats while theirs was with dairy cattle. Second, the goats were given injections only during eating of a meal, not continuously for eight hours. Third, the injection rate was greater in our experiments per unit metabolic mass (1.2 versus 0.18 mmoles per minute). Although we have not tested for the temperature effect of sodium acetate given intravenously, acetic acid given intravenously (0.14 mmoles per minute per $BWkg^{.59}$), a level similar to that used by Dowden and Jacobson, for 120 minutes caused an elevated hypothalamic temperature 10.6 C beginning after about 30 minutes of injection (5). Since goats in the present experiment ate for only 5 to 10 minutes per meal, it is probable that little temperature change was caused. We would suggest that the high rate and long duration of the injections of Dowden and Jacobson probably caused toxic levels of blood acetate. They alluded to this possibility in a later paper (14).

Our experiment [illegible] from that of Holder's (13) with sheep [illegible] for 210 minutes and fed once daily [illegible], in that [illegible] were injected only while they were eating and being fed ad libitum. Perhaps experiments with ad libitum feeding have more physiological significance than those with controlled feeding when the object is to define the control of hunger and satiety on a meal basis. Feedback signals are more likely to be suppressed through conditioning to permit long-term energy balance on once or twice daily feeding schedules. In our experiment, the blood acetate concentrations during the intravenous injections reached levels several times higher than those found anywhere in the goat except in the rumen [illegible]. In Holder's experiment the acetate concentration of the jugular blood was made as high as or higher than that which it reached postprandially. His experiment did not necessarily test for a receptor in the ruminal venous drainage system, since the concentration of acetate in the ruminal vein may sometimes reach a level higher than that caused by his intravenous injection of acetate. The experiment probably was a satisfactory test for a hypothalamic receptor.

although the feeding schedule used may have eliminated this response.

Although our experiment, as other acetate injection experiments, does not conclusively indicate that the monitoring of acetate is a component of the satiety mechanism in ruminants, it does seem apparent that there is a receptor sensitive to changes in acetate concentration. Most of the results of this type of experiment can be accounted for by the existence of receptors in the ruminoreticulum on the lumen side sensitive to at least acetate and possibly other volatile fatty acids.

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References

(1) Annison, E. F., K. J. Hill, and D. Lewis. 1957. Studies on the portal blood of sheep. 2. Absorption of volatile fatty acids from the rumen of the sheep. Biochem. J., 66: 592.

(2) Baile, C. A., and A. W. Mahoney. 1967. Hypothalamic function in ruminant food intake regulation. Proc. Seventh Intern. Congr. Nutrition, 2: 67.

(3) Baile, C. A., A. W. Mahoney, and J. Mayer. 1967. Preliminary report on hypothalamic hyperphagia in ruminants. J. Dairy Sci., 50: 1151.

(4) Baile, C. A., and J. Mayer. 1967. Intragastric injections of liquid diet, water, and acetate and meal patterns of goats. Amer. J. Physiol., 213: 387.

(5) Baile, C. A., and J. Mayer. 1968. Hypothalamic temperature and the regulation of food intake in goats. Amer. J. Physiol., 214 (5): 677.

(6) Baile, C. A., and W. H. Pfander. 1966. A possible chemosensitive regulator mechanism of ovine feed intake. Amer. J. Physiol., 210: 1243.

(7) Baumgardt, B. R. 1964. Practical observations on the quantitative analysis of free volatile fatty acids (VFA) in aqueous solutions by gas liquid chromatography. Univ. Wis. Dept. Dairy Sci., Bull. 1.

(8) Bensadoun, A., O. L. Paladines, and J. T. Reid. 1962. Effect of level of intake and physical form of the diet on plasma glucose concentration and volatile fatty acid absorption in ruminants. J. Dairy Sci., 45: 1203.

(9) Conrad, H. R. 1966. Symposium on factors influencing the voluntary intake of herbage by ruminants: Physiological and physical factors limiting feed intake. J. Anim. Sci., 25: 227.

(10) Cook, R. M., and L. D. Miller. 1965. Utilization of volatile fatty acids in ruminants. I. Removal of them from portal blood by the liver. J. Dairy Sci., 48: 1339.

(11) Dowden, D. R., and D. R. Jacobson. 1960. Inhibition of appetite in dairy cattle by certain intermediate metabolites. Nature, 188: 148.

(12) Hoffman, W. S. 1937. A rapid photoelectric method for the determination of glucose in blood and urine. J. Biol. Chem., 120: 51.

(13) Holder, J. M. 1963. Chemostatic regulation of appetite in sheep. Nature, 200: 1074.

(14) Jacobson, D. R., S. B. Carr, and T. Sutardi. 1967. Appetite regulation in the ruminant. Proc. Seventh Intern. Congr. Nutrition, Pergamon Press, New York, 2: 63.

(15) Manning, R., G. L. Alexander, H. M. Krueger, and R. Pogart. 1959. The effect of intravenous glucose injections on appetite in adult ewes. Amer. J. Vet. Res., 20: 242.

(16) Montgomery, M. J., L. H. Schultz, and B. R. Baumgardt. 1963. Effect of intraruminal infusions of volatile fatty acids and lactic acid on voluntary hay intake. J. Dairy Sci., 46: 1380.

(17) Ramsey, H. A., and C. L. Davis. 1965. Metabolism of N-butyrate by the adult goat. J. Dairy Sci., 48: 381.

(18) Rook, J. A. F., C. C. Balch, and R. C. Campling. 1960. The effects of intraruminal infusions of acetic, propionic and butyric acids on nitrogen retention in growing heifers. Proc. Nutrition Soc., 19: 4.

(19) Simkins, K. L., Jr., J. W. Suttie, and B. R. Baumgardt. 1965. Regulation of food intake in ruminants. 4. Effect of acetate, propionate, butyrate, and glucose on voluntary food intake in dairy cattle. J. Dairy Sci., 48: 1635.

(20) Ulyatt, M. J. 1965. The effects of intraruminal infusion of volatile fatty acids on food intake of sheep. New Zealand J. Agr. Res., 8: 397.

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Depression of feed intake of goats by metabolites injected during meals

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BAILE, CLIFTON A., AND JEAN MAYER. *Depression of feed intake of goats by metabolites injected during meals.* Am. J. Physiol. 217(6): 1830–1836, 1969.—To test the relative depression of feed intake by various metabolites, goats were maintained in stalls with feeders equipped with photoelectric relays to activate pumps and recorders during spontaneous meals. Sodium acetate, propionate, butyrate, a mixture of the three salts (VFA) (pH = 6.5), and propionic acid were injected intraruminally at rates of 2.5, 5, 10, or 20 mmoles/min during eating. All solutions depressed feed intake although butyrate was least effective. Regression equations expressing the relationship between per cent depression of feed intake relative to control periods (Y) and average mmoles of salt injected per meal (X) were: for acetate, $Y = .39X - 5.18$, $r = .76$, $P < .01$; for propionate, $Y = .36X - .27$, $r = .60$, $P < .01$; for butyrate, $Y = .17X + 11.40$, $r = .50$, $P < .02$; and for VFA, $Y = .38X + 8.74$, $r = .70$, $P < .01$. Intraruminal injection of sodium lactate (pH = 6.5) was strong but variable while sodium formate (pH = 6.5) was relatively weak as a depressant. Glucose, $NaHCO_3$, or mixtures of the two caused only small depressions. Because volatile fatty acids are the main products of fermentation in the rumen and because their production rates increase with feeding, the feeding-depressant effects of acetate and propionate may be important in regulation of energy balance in ruminants especially when grain rations are fed.

feedbacks for control of feed intake; feed intake control; ruminants, volatile fatty acids

EXPERIMENTS WITH RUMINANTS, e.g., goats and sheep, have shown that they control their feed intake to maintain energy balance under many conditions (1, 7, 16, 17, 19, 23), but not when fed some roughage rations (14, 15). Lesions in the ventromedial area of the hypothalamus result in hyperphagia (8), and lesions in the lateral areas of the hypothalamus cause aphagia and adipsia (3). Although ruminants are similar to monogastric animals in these aspects, the feedbacks and receptor sites for the control of feeding may be different. Under our laboratory and feeding conditions, neither hypothalamic temperature nor heat loss changes (6), glucose utilization rate (4), nor ruminoreticular distension (9) is an apparently important feedback. Volatile fatty acids may act as important feedbacks in the control of feed intake in ruminants (1). They are produced by microbial fermentation in the ruminoreticulum, a large proportion are absorbed directly from the rumen, and they are the most important energy source for ruminants.

The present experiments were designed to characterize more specifically the depression of feed intake of several intermediary metabolites of the rumen. A system was [illegible] so that goats fed ad libitum could be injected intraru[illegible]nally with test solutions during spontaneous meals. Def[illegible] differences in feed intake depression by the metabo[illegible] were shown under the conditions tested.

MATERIALS AND METHODS

Six goats (30–40 kg) were used in these studies, each prepared surgically with a ruminal fistula and fitted with a cannula of hard rubber. The goats were housed in a room with constant lighting and maintained at 24 ± 1 C. They were fed a concentrated diet ad libitum (less than 9% fiber, Omelene, Ralston Purina). The feed and water were measured and the orts determined between 8:00 and 9:00 AM each day. Also, at this time, the treatments were initiated, and the amounts of test solutions injected intraruminally during the preceding 24 hr were determined. The goats were locked in stanchions with an apparatus in front of them that monitored their feeding behavior (5). When a goat ate from its feed container, a light beam impinging on a photocell was broken which activated a relay that started a pump; thus the goats were injected intraruminally during spontaneous meals.

In all experiments the duration of each treatment was 2 days preceded by a 2-day control period and followed by a 2-day postinjection period during which no injections were given. Except in the case of some of the treatments of *experiment II*, all treatments of each experiment were replicated on each animal. The intraruminal injection rate of all solutions tested was 5 ml/min.

Experiment I. Goats 4, 5, and *6,* during the 2-day injection period, were given 2.5, 5, 10, or 20 mmoles/min of sodium acetate (pH = 6.5, Fisher NF grade) while eating. The concentration of acetate used was 0.5, 1.0, 2.0, or 4.0 M.

Experiment II. The treatments tested were 2.5, 5, 10, or 20 mmoles/min of propionic acid (Fisher certified grade) or sodium propionate (Fisher purified) (pH = 6.5). The 10- and 20-mmoles/min treatments of the acid and the sa[illegible] were not replicated, whereas the other two treatments were replicated. *Goats 1, 2,* and *3* were used.

Experiment III. In this experiment, the same levels of injection as those of *experiment I* were given, but solutions of sodium butyrate (pH = 6.5, Fisher reagent grade) were used. *Goats 1, 2,* and *3* were tested.

Experiment IV. A mixture of the sodium salts (pH = 6.5) of acetate (55%), propionate (30%), and butyrate (15%) was injected intraruminally so that during spontaneous

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...l either 2.5, 5, 10, or 20 mmoles/min were given on treatment days. The proportions of the salts were similar to those measured in the rumen fluid on control days. *Goats 2* and *7* were used.

Experiment I'. Sodium formate (pH = 6.5, Fisher certified) was injected at 10 mmoles/min in *goats 1, 2,* and ... Sodium lactate (pH = 6.5, Fisher) was injected intraruminally at 5 and 10 mmoles/min in *goats 3, 4,* and *5.* Treatments were replicated on each of the goats.

Experiment II. Goats *4, 5,* and *6* were given, on treatment days, an intraruminal injection of *1*) 6.7 mmoles/min of glucose, *2*) 6.7 mmoles/min of glucose plus 5 mmoles/min sodium bicarbonate (Fisher certified), or *3*) 5 mmoles/min sodium bicarbonate. The glucose provided the carbon equivalents of the 20-mmoles acetate treatment of *experiment I.*

A meal was defined as having at least 5 min duration, and at least 30 min must have elapsed since a previous meal. The goats broke the light beam across the feeders only infrequently between the so-defined meals, and such occurrences were disregarded in determining meal patterns. The length of the meal was measured from the recorder charts and included the intermittent time the goat may have had its head out of the feeding container during a meal.

During experiments, rumen fluid samples were taken daily on control and on treatment days. Samples were obtained prior to the feeding of fresh feed to goats in the morning. Samples were placed in a test tube containing ... the pH was determined, and the samples were frozen. Samples were later prepared for volatile fatty acid analysis by gas-liquid chromatography (Hewlett Packard ... column gas chromatograph, model 402) by the method of Baumgardt (12). In the preparation of each sample, a known amount of acetate-^{14}C (New England Nuclear Corp.) was added prior to the extraction procedure. Although the labeled acetate contributed an insignificant amount of acetate to the sample, the ^{14}C activity was determined and a recovery efficiency calculated. The acetate-^{14}C activity was counted in a scintillation system (... 702 series scintillation counter, Nuclear-Chicago).

Feed and water intakes, meal frequencies, and meal lengths were tested for significance by paired *t* tests (control-period data were paired with the treatment period data of each animal). The rumen fluid pH, total concentration of volatile fatty acids, and component proportions of the volatile fatty acids were tested for differences by using unpaired *t* tests of control and treatment data.

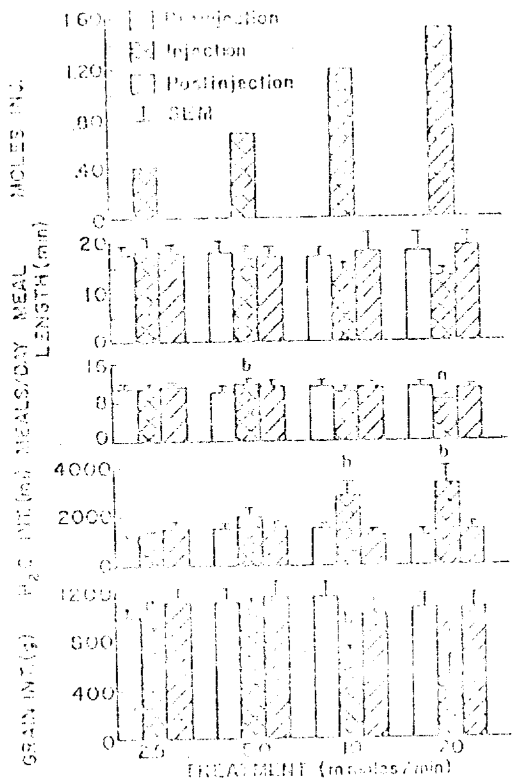


FIG. 1. *Experiment I.* Na acetate (pH ...) was injected intraruminally into goats during spontaneous meals. Data are all presented as daily means of 3-day periods. Letters a, b, and c denote levels of significance .05, .01, and .001, respectively, of the difference between the preinjection and injection means as tested by paired *t* tests.

of feed intake with increasing rates of injection with one exception (Figs. 1, 4). We have no explanation other than chance for the apparent trend toward a greater feed intake with injections of 5 mmoles than with 2.5 mmoles/min of acetate. The 5-mmoles/min treatment and the following effects: water intake was slightly increased and the rumen pH was increased (*P* < .05, Table 1); concentration of volatile fatty acids of the rumen fluid and the number of meals were increased (*P* < .05), and although the proportion of acetate in the rumen fluid did not increase (*P* > .10), volatile fatty acid concentrations did.

There was no difference between the effect on feed intake of sodium propionate (pH = 6.5) and propionic acid (*P* > .25), tested by a paired *t* test. Sodium propionate injections caused an increased oral water intake with increased ...

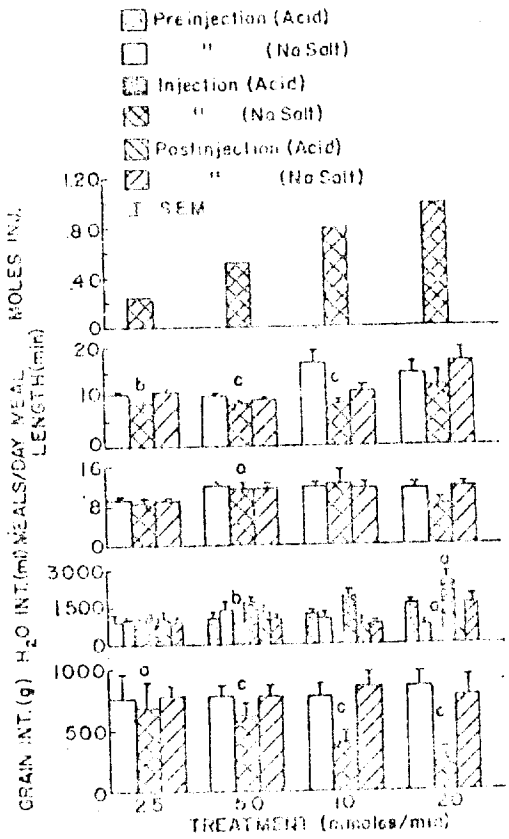


FIG. 2. *Experiment II.* Na propionate (pH = 6.5) or propionic acid was injected intraruminally during meals of goats fed ad libitum. Effect of acid and salt differed only in water-intake response and therefore data for other parameters are combined. All data are daily means of 2-day periods. Denotations of significance are same as in Fig. 1.

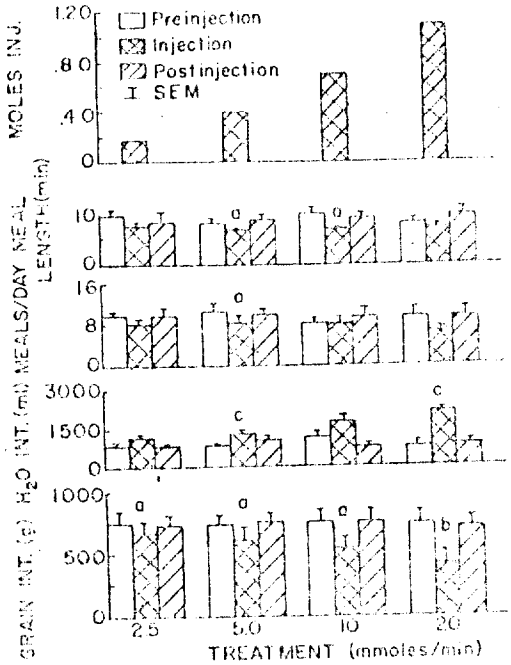


FIG. 3. *Experiment III.* Na butyrate (pH = 6.5) was injected intraruminally during spontaneous meals in goats. All data are daily means of 2-day periods. Denotations of significance are the same as in Fig. 1.

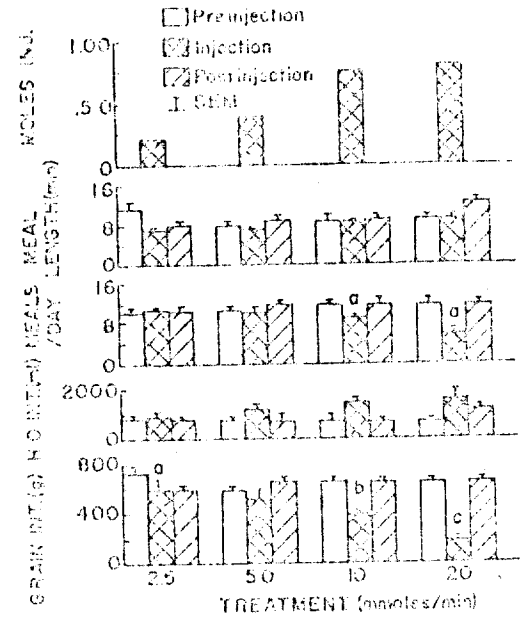
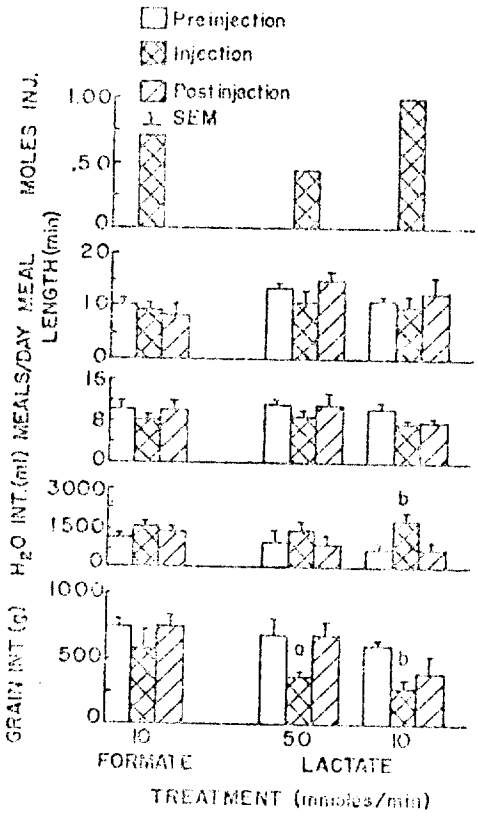


FIG. 4. *Experiment IV.* Solutions (pH = 6.5) of a mixture of sodium acetate (55%), propionate (30%), and butyrate (15%) were injected intraruminally into goats during spontaneous meals. Data are expressed as daily means of 2-day periods. Denotations of significance are same as in Fig. 1.

mmoles/min during eating. Lactate, on the other hand, was a strong depressant of feed intake relative to the other metabolites tested. The response was quite variable, but in some trials the lactate became toxic. Unlike the recovery from the response from acetate, propionate, or butyrate, the recovery from the higher rate of lactate was especially slow. The injection of neither 6.67 mmoles/min of glucose nor of 6.67 mmoles/min of glucose plus 5 mmoles $NaHCO_3$ alone caused a significant depression of feed intake (Fig. 6).

Rumen fluid data. The pH of the rumen fluid increased with increasing rates of injection of all the solutions tested except glucose (Table 1). During the injection of the volatile fatty acids the total concentration of these acids of the rumen fluid tended to increase at the slower rates of injection and to decrease with the higher levels relative to the control periods. The molar proportion of acetate, propionate, or butyrate, when injected alone during an experiment, tended to increase in proportion to the other acids. This was especially true for butyrate for which the proportion nearly tripled during the highest rate of butyrate injection. Although the pH of the rumen fluid increased markedly ($P < .01$) with increasing rates of injection of the mixture of acetate, propionate, and butyrate, neither the average total concentration of volatile fatty acids nor the molar proportions changed appreciably with different treatments. Neither total volatile fatty acid concentration nor molar proportion was significantly affected by the injections of glucose, although the total volatile fatty acid concentration and propionate proportion tended to increase and the acetate proportion to decrease.



3. *Experiment I.* Solutions of sodium formate or lactate (pH ... re injected intraruminally during spontaneous meals. Denota... f significance are same as in Fig. 1.

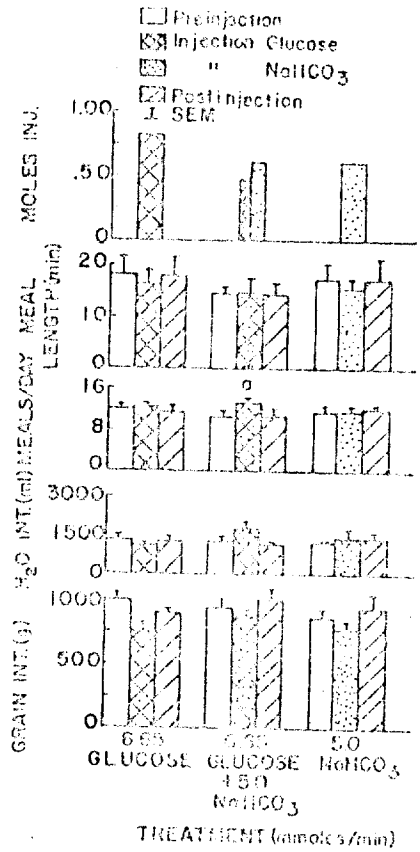


FIG. 6. *Experiment II.* Solutions of 1.33 M glucose, 1.33 M glucose plus ... M $NaHCO_3$ or ... M $NaHCO_3$ were injected intraruminally to goats during spontaneous meals. All data are presented as daily means of 2-day periods. Denotations of significance are same as in Fig. 1.

There were no apparent differences between the effects of the injections of propionate or of propionic acid on pH, molar proportion, or concentration of the volatile fatty acids of the rumen fluid. The pH of the rumen fluid tended to increase with increasing rate of propionate injection although insufficient data were collected to estimate pH or concentrations of the volatile fatty acids of the rumen fluid of goats during the 20-mmoles/min treatment. The concentration of volatile fatty acids tended to decrease with increasing rates of propionate injection; changes in the proportion of the different acids of the rumen fluid were not marked.

DISCUSSION

The data from these experiments clearly show that a close relationship exists between the rate of injection of acetate, propionate, or a mixture of volatile fatty acids and the depression of feeding. The rate of production of acetate, propionate, and butyrate in the rumen under fed conditions has been reported to be about 1.23, 1.17, and 0.97 mmoles/min, respectively, for sheep (22). Therefore, the injection rates of the present experiments are probably near the physiological rates of production following introduction of readily fermentable foodstuffs, except perhaps the 20-mmoles/min treatment. It is quite likely that the feeding depressant effect of the volatile fatty acids may play a significant role in the overall regulation of energy balance since 1) they are important energy sources to the ruminant, 2) their rate of production increases with feeding, and 3) they are the first and most immediate products of the digestive process to be absorbed. Also produced in the rumen in substantial amounts, at least under certain conditions, are lactate (27, 28) and formate (11). These metabolites are not usually very important energy sources for ruminants, but lactate especially is produced at high rates for a short period of time after readily digestible carbohydrates are ingested (27). On these bases the tests with formate and lactate were done. Glucose is readily fermented in the rumen and was of interest in the present experiments as a test of specificity of the other intermediary metabolites.

These experiments differ from some previous tests of the effect of intermediary metabolites on feeding in ruminants in that the injections were given early during eating of spontaneous meals. In experiments with goats (5, 7, 9) and sheep (10) receiving injections of acetate during spontaneous meals, depressions of feed intake were comparable when only the doses were tested. Experiments with propionate and lactate intraruminally over a feeding period lasting several hours caused feeding depression in sheep (13, 14), whereas tests with goats have failed to show this effect (11, 12). Although sodium propionate injections caused [illegible] (12). It has been suggested that [illegible] and containing both acetate and propionate [illegible]

TABLE 1. *Data from rumen fluid samples*

Experiment	Treatment	N	pH	VFA mM	Acetate %	Propionate %	Butyrate %
Acetate	0	36	5.55	85	62	25	13
	2.5	10	5.65	85	67‡	26*	10‡
	5	9	5.92‡	93‡	56	31	13
	10	16	6.22‡	71	67‡	24‡	9‡
	20	8	6.51‡	60‡	64‡	25‡	12
Propionate	0	12	5.93	82	55	32	13
	2.5	9	5.93	89	57	28	15
	5	11	5.46*	67‡	55	34	12
	10	4	6.04‡	71	52	36	12
	20						
Butyrate	0	27	5.56	80	52	35	13
	2.5	7	5.84‡	87*	53	31	15
	5	9	6.17‡	50*	51	29‡	17*
	10	8	6.01‡	71	48‡	27‡	25*
	20	10	6.41‡	67‡	45‡	24‡	31‡
VFA	0	15	6.05	69	58	31	11
	2.5	7	5.93	93	59	32	10
	5	8	6.12‡	61	62	25	13
	10	7	6.06‡	79	62	23	11
	20	8	6.30‡	61	67‡	24*	9
Formate	0	8	5.62	71	59	32	9
	10	11	6.51‡	68	57	31	12*
Lactate	0	12	5.72	72	56	26	14
	5	7	6.03‡	53*	57	28	15
	10	7	6.74‡	51*	55	31*	16*
Glucose	0	15	5.63	77	51	35	14
	6.67	11	5.53	95*	45	40	14
Glucose + $NaHCO_3$	6.67	9	5.75	91*	49	42	9
$NaHCO_3$	5.0	13	6.26‡	75	59	29	12*

Treatments are designated by millimoles per meal injected. * $P < .10$, level of significance between the 0 and injection treatments (2 tests). ‡ $P < .05$. ‡ $P < .01$.

depression of feed intake in dairy cattle (13), which is in contrast to the effects observed with a mixture of volatile fatty acids in studies on sheep (18), or dairy cattle (29, 31), or in the present studies with goats. Those injections resulting in no depression were given in one dose 1 hr prior to a feeding period, and the cattle had been conditioned to a single 6 hr feeding period per day.

Previous tests with butyrate (24, 29, 31, 32) and lactate (30, 24) injected intraruminally during a daily feeding period showed a small depressant effect in cattle and sheep fed mainly roughages. Our experiments differ both in that the injections were given during spontaneous meals and a grain ration was fed. Formate injections into the stomachs of sheep in a single dose did not depress feeding (25, 26).

Volatile fatty acid concentration of the rumen fluid generally decreased within a varying degree of feeding depression. From the data of Table 1 it is not possible to determine the change in total the fatty acid concentration of the rumen fluid during a meal, but it can be concluded that the depressant effect was not due to a general elevation of usage or of total volatile fatty acids between meals, since the samples were taken after the feed was removed. The only sustained elevation, apparently, was during meals and because of decreased feed intake, the average concentration of volatile fatty acids in the rumen fluid decreased between meals. Butyrate was not as effective a depressant even though there was a sustained increase of butyrate concentration of the rumen fluid. The increase in concentration may have been the result of the more nearly normal feed intake, and of the slower uptake or metabolism of butyrate than of acetate or propionate. The proportion of acetate increased during *experiment I* although the concentration of acetate was not changed. During *experiment II*, although the proportion of acids was little affected, the acetate proportion tended to increase. The pH of the rumen fluid increased as increasing amounts of Na^+ were injected. In no case was there evidence that the goats developed sustained unphysiological levels of rumen fluid volatile fatty acids or rumen fluid pH.

Although the injection of Na^+ resulted generally in increased oral water intake, the feed intake depression by the sodium salts of organic acids was apparently not related to the increased Na^+ level. Solutions of propionic acid or sodium propionate had similar feeding depressing effects. During propionic acid treatments the oral water intake was decreased. Other evidence is the lesser depressant effects of solutions of sodium butyrate and $NaHCO_3$ per millimole injected as compared with acetate and propionate. Previous experiments have also shown the depressant effect of the volatile fatty acids not to be simply an osmotic effect (10, 33). We have recently shown that the increased oral water intake would not inhibit feed intake of goats fed a concentrated diet (9).

The regression lines and correlation coefficients of the percent depression of feed intake and the millimoles injected per meal were calculated. Percent depression of the treatment period as compared with the intake of the control was selected in that the feed intakes of each animal served as its own control. The millimoles injected per meal were calculated by dividing the moles injected for the 2-day treatment period by the number of meals. The latter parameter is useful in that it is an expression of the effectiveness of the metabolite as a function of meal pattern, and the individual goat's meal pattern is taken into account. The regression equations and correlation coefficients of the treatments were: for acetate $Y = .39X - 5.18$, $N = 24$, $r = .76$, $P < .01$; for propionate $Y = .36X - .27$, $N = 13$, $r = .59$, $P < .01$; for butyrate $Y = .17X + 11.43$, $N = 24$, $r = .50$, $P < .02$; for VFA (mixture of acetate propionate, butyrate) $Y = .33X + 8.74$, $N = 16$, $r = .79$, $P < .01$; for lactate $Y = 29 + .30X$, $N = 8$, $r = .30$, $P > .1$; for formate $Y = 26 + .03X$, $N = 6$, $r = .02$, $P > .9$; for glucose $Y = 15 + .39X$, $N = 6$, $r = .27$, $P > .05$; for glucose plus $NaHCO_3$ $Y = .35X - 18$, $N = 6$, $r = .52$, $P > .3$. It can be seen that the slopes of the treatments of acetate, propionate, or volatile fatty acid mixture are all similar while butyrate is clearly less.

In Fig. 7 are shown the regression lines for the millimoles injected per meal and percent depression of feed intake corrected for the estimated digestible energy of the injection solutions (the heat of combustion of the free acids or the solutions injected was converted to estimated equivalents of feed, the digestible energy of the feed was estimated to be 3 kcal/g). The slopes of these lines were less than those of the respective lines of the uncorrected feed-intake

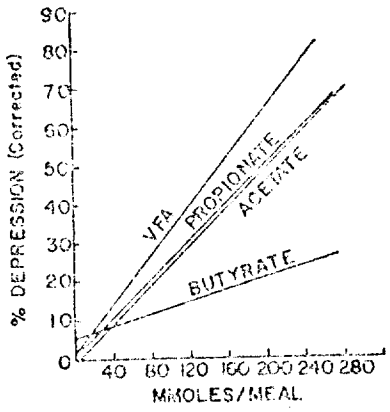


FIG. 7. Regression lines are shown of percent depression of 2-day injection periods corrected for estimated digestible energy of the salt injected as compared with 2-day preinjection periods versus millimoles salt injected per meal. For acetate: $Y = .25X - 1.67$, $N = 24$, $r = .51$, $P < .02$; for propionate: $Y = .25X - 2.37$, $N = 18$, $r = .67$, $P < .02$, for butyrate: $Y = .08X + 5.1$, $N = 24$, $r = .27$, $P > .10$; for VFA (mixture of acetate, propionate, butyrate): $Y = .312X + 4.15$, $N = 16$, $r = .74$, $P < .01$.

data. The correlation coefficient of the butyrate data was not significantly different from 0. Figure 7 clearly shows the relationship of ruminal intermediary metabolites and feeding and that the depression of food intake is not merely a compensation for the energy of the solutions injected. These regression equations are, of course, applicable only to the conditions under which these tests were done, although it is quite probable that butyrate would not be as strong a depressant as acetate, propionate, or a mixture of volatile fatty acids injected intraruminally independent of the type of ration fed. The small butyrate depression observed in *experiment III*, after correcting for the digestible energy injected, was comparable to that observed with $NaHCO_3$.

Acetate and propionate have nearly the same effect whereas, again, butyrate is quite ineffective. We postulated that there were receptors on the lumen side of the rumen that were sensitive to acetate concentration changes of the rumen fluid since intravenous injections of acetate are much less of a depressant than intraruminal injections (5). We have further shown that an increase in the concentration of acetate in the rumen fluid but with only a small change in volume, depresses feed intake more than just an increase in the quantity of acetate and an increase in overall volume of the rumen (9). In contrast, we have found that propionate injection in the jugular vein during spontaneous meals is a more effective feeding depressant than intraruminal injections (2). It is also true that injections in the ruminal vein cause a greater depression than similar injections in the jugular vein (2). This is evidence that goats may sense the body state of propionate differently than that of acetate; there may be receptors (possibly in the liver) sensitive to propionate in addition to possible receptors in the rumen wall. These experiments clearly show that acetate and propionate reduce feeding when only small amounts are added to the rumen. Butyrate injected intraruminally is quite ineffective as a depressant, and therefore apparently the sensing mechanisms for acetate and propionate are not very sensitive to butyrate.

Although lactate was a strong depressant of feeding, glucose injections with or without $NaHCO_3$ did not cause a significant depression, especially when the estimated digestible energy of the glucose was taken into account. Glucose would be expected to cause first an increased production of lactate and second of the volatile fatty acids (20, 27, 33). The purpose of the glucose plus $NaHCO_3$ solution was to buffer the possible decreased pH following fermentation of glucose. Under the conditions of these experiments introduction of lactate did not cause a sustained increase in the proportion of butyrate as observed in a previous experiment (24).

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REFERENCES

1. BAILE, C. A. Regulation of feed intake in ruminants. *Federation Proc.* 27: 1361–1366, 1968.
2. BAILE, C. A. Propionate as a possible receptor for control of meal size in ruminants (Abstract). *Federation Proc.* 28: 491, 1969.
3. BAILE, C. A., A. W. MAYHEW, AND J. MAYER. Induction of hypothalamic [illegible] and adipsia in goats. *J. Dairy Sci.* 51: 1473–1485, [illegible]
4. BAILE, C. A., AND J. MAYER. Effects of insulin-induced hypoglycemia and hyperglycemia on eating behavior in goats. *J. Dairy Sci.* 51: 1479–1484, 1968.
5. BAILE, C. A., AND J. MAYER. Effect of intracerebral versus intravenous [illegible] infusion of acetate on feed intake of goats. *J. Dairy Sci.* 51: 1490–1496, 1968.
6. BAILE, C. A., AND J. MAYER. Hypothalamic lesions, prolactin, and the regulation of feed intake in goats. *Am. J. Physiol.* 215: 1059–1064, 1968.
7. BAILE, C. A., AND J. MAYER. Intracerebral injections of hypertonic water and acetate and food intake of goats. *Am. J. Physiol.* 217: 707–712, 1969.
8. BAILE, C. A., J. MAYER, A. W. MAHONEY, AND C. L. MCLAUGHLIN. Hypothalamic hyperphagia in goats and some of its variations on its effect on glucose utilization rate. *J. Dairy Sci.* 52: 101–103, 1969.
9. BAILE, C. A., J. MAYER, AND C. M. LAUGHLIN. Feeding behavior of goats: ruminal distension, ingesta dilution, and acetate concentration. *Am. J. Physiol.* 217: 397–402, 1969.
10. BAILE, C. A., AND W. M. PEARSON. A possible chemosensitive [illegible] controlling [illegible] of some feed intake. *Am. J. Physiol.* [illegible] 1143–1148, 1969.
11. BAILE, C. A. A determination of liquid scintillation counting efficiency by pulse height shift. *Intern. J. Appl. Radiation Isotopes* 3: 1, 1961.
12. BRAWNER, R. R. Preliminary observations on the quantitative analysis of free volatile fatty acids. VFA in rumen [illegible] and liquid chromatography. *Bull. Dairy Sci. [illegible] J. Lab.*
13. BRAYDEN, A. N., AND R. C. WARNER. Effect of intravenous infusion of dipropionate, propionate, and butyrate on food intake in dairy [illegible] *J. Dairy Sci.* [illegible] 1951.
14. [illegible] Reversal of [illegible] by [illegible] *J. Dairy Sci.* [illegible]

16. BLAYLOCK, R. L., AND R. S. WILSON. Voluntary intake of concentrates by steers. *Animal Prod.* 4: 351–358, 1962.
17. CAMPLING, R. C. A preliminary study of the effect of pregnancy and lactation on the voluntary intake of roughage by cows. *Proc. J. Agr.* 20: 25–29, 1963.
18. COWLEY, R. L., AND M. J. MONTGOMERY. Effect of varying roughage to concentrate ratio of isonitrogenous rations on food intake by ruminants. *J. Dairy Sci.* 42: 1463–1467, 1963.
19. EVANS, A. B. Nutritional studies on food intake and digestion in sheep. V. Effects of intraruminal infusions of acetic acid on voluntary intake of roughages of sheep. *Australian J. Agr. Res.* 17: 741–755, 1966.
20. HEIDELBERGER, G., AND W. HOLMES. Studies of feed intake and utilization by sheep. I. The voluntary food intake of dry pregnant and lactating ewes. *J. Agr. Sci.* 71: 273–280, 1968.
21. HUNGATE, R. E. Quantitative aspects of the rumen fermentation. In: *Physiology and Biochemistry of the Rumen*, edited by R. W. Dougherty. Washington, D.C.: Butterworths, 1966, p. 43.
22. HUNGATE, R. E., R. W. DOUGHERTY, M. P. BRYANT, AND R. M. COTTA. Microbiological and physiological changes associated with acute indigestion in sheep. *Cornell Vet.* 52: 423–448, 1962.
23. LANG, R., AND A. J. LOUGHER. Measurement of the rates of production of acetic, propionic and butyric acids in the rumen of sheep. *Irish J. Agr.* 2: 401–414, 1963.
24. MONTGOMERY, M. J., AND E. L. EVERHART. Regulation of food intake in ruminants. I. Pelleted rations varying in energy concentration. *J. Dairy Sci.* 47: 574–580, 1964.
25. MONTGOMERY, M. J., E. H. SCHEITZ, AND B. E. BAUMAN. Effect of intraruminal infusion of volatile fatty acids and lactic acid on voluntary hay intake. *J. Dairy Sci.* 46: 1350–1354, 1963.
26. NEWBERG, H. On the areas of the stomach of sheep that are sensitive to formic acid and histamine. *J. Agr. Sci.* 60: 295–305, 1963.
27. NEWBERG, H., AND A. TAYLOR. The effect of histamine and with formic or acetic acids on food intake and rumen motility when infused into the omasum of sheep. *J. Agr. Sci.* 71: 295–299, 1968.
28. PHILLIPSON, A. T., AND R. A. MCANALLY. Studies on the carbohydrates in the rumen of the sheep. *J. Exp. Biol.* 6: 239–254, 1933.
29. ROOK, J. A. F., C. C. BALCH, R. C. CAMPLING, AND L. J. I. The utilization of acetic, propionic and lactic acids by heifers. *Brit. J. Nutr.* 17: 399–409, 1963.
30. SMITHS, K. L., J. W. SMITH, AND B. R. BAUMGARDT. Regulation of food intake in ruminants. I. Effect of acetate, propionate, fat and glucose on voluntary intake in dairy cattle. *J. Dairy Sci.* 52: 1962.
31. THOMAS, J. W., L. A. MOORE, M. OLAMOTO, AND J. R. SA. Study of factors affecting rate of intake of heifers fed silage. *Dairy Sci.* 44: 1471–1483, 1961.
32. UPTAGRAFT, M. J. Studies of some factors influencing food intake in sheep. *Proc. New Zealand Soc. Animal Prod.* 24: 43–56, 1964.
33. UPTAGRAFT, M. J. Effects of intraruminal infusions of volatile fatty acids on food intake of sheep. *New Zealand J. Agr. Res.* 9: 1066–1069.
34. WALTER, D. R., AND L. H. SCHULTZ. Lactic acid production in the rumen. *J. Dairy Sci.* 39: 1353–1359, 1956.
35. WESTON, R. H. Factors limiting the intake of feed by sheep. The significance of protozoa in the capacity of the abdomen to handle digesta, and the supply of glucogenic substrate. *Australian J. Agr. Res.* 17: 939–951, 1966.

J. Appl. Physiol. 4: 797-799, 1952

Effect of Acetate on Tm_{PAH} in Man

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CROSS AND TAGGART (1) have shown in studies on excised rabbit kidney that acetate is importantly involved in the tubular transport of *p*-aminohippuric acid (PAH). Mudge and Taggart (2, 3) have reported that during the intravenous infusion of sodium acetate or lactate in the dog, Tm_{PAH} may be substantially increased above control values, while Schachter and Freinkel (4) find that the self-depression of Tm_{PAH} in the dog caused by overloading at high plasma PAH concentrations can be prevented, or reversed after depression by the intravenous administration of sodium acetate.

The present study reports observations on the effects of acetate on Tm_{PAH} in man.

METHODS

Inulin was determined by a modification of Harrison's method as described by Goldring and Chasis (5), PAH by the method of Smith, Finkelstein, Aliminosa, Crawford and Graber (6), and creatinine by the Bonsnes and Taussky method (7).

Of seven subjects examined, four were without renal disease, two had essential hypertension, and one had chronic glomerulonephritis. Observations were made in the postabsorptive state at relatively constant plasma concentrations of inulin and PAH maintained by constant intravenous infusion. After three control periods for the measurement of Tm_{PAH}, an infusion of 0.5 M sodium acetate was administered at a rate, varying in different subjects, from 0.08 to 0.10 mEq/kg/minute; after a 15- to 30-minute discard period, Tm_{PAH} was again determined in three consecutive [illegible]-minute urine collection periods during acetate infusion. When acetate was administered all subjects complained of fullness in the head, nausea and palpitation; [illegible] vomited and in one instance carpo pedal spasm occurred after 740 cc. of 0.5 M [illegible]um acetate had been given over a period of 45 minutes. Plasma concentrations [illegible] PAH were maintained such that the load/Tm_{PAH} ratio approximated 2 to 3, the [illegible]ma concentration remaining relatively constant during the control and experimental periods.

Exogenous creatinine/inulin clearance ratios (C_{cr}/C_{in}) before and during acetate [illegible]sion were also determined in three subjects without renal disease, using constant [illegible]ma creatinine concentrations varying from 7 and 11.0 mg/100 cc. in different [illegible]jects. After three control periods, sodium acetate (0.5 M) was infused at a rate [illegible]ying from 0.131 to 0.2[illegible] mEq/kg/minute and after a 30-minute discard

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period, three consecutive 15-minute observations were made during the acetate infusion.

Results

The effects of the sodium acetate on Tm_{PAH} in seven subjects are recorded in table 1. In each instance the control Tm_{PAH} and acetate Tm_{PAH} represent the average of three urine collection periods. Of the four subjects without renal disease, three

TABLE 1. EFFECT OF SODIUM ACETATE ON Tm_{PAH} IN MAN

Subject				Tm_{PAH}[1]			
Age	Sex	Diagnosis	Rate of acetate infusion	Control	Acetate	Av. Acetate / Control	Max. Acetate / Control
yr.			*mEq./kg./min.*	*mg./min.*	*mg./min.*		
22	F	Osteomyelitis	0.19	49.5	71.3	1.44	1.6[illegible]
40	M	Alcoholism	0.15	57.7	74.9	1.30	1.5[illegible]
36	M	Pneumonia	0.08	73.2	84.2	1.15	1.[illegible]
39	M	Peptic ulcer	0.13	94.0	95.4	1.01	1.[illegible]
9	F	Essential hypertension	0.09	81.0	105.0	1.26	1.[illegible]
53	F	Essential hypertension	0.09	57.9	58.0	1.00	1.00
10	F	Glomerulonephritis	0.17	42.6	60.8	1.43	1.4[illegible]

[1] Data on Tm_{PAH} are corrected to 1.73 sq. m. body surface area.

TABLE 2. EFFECT OF SODIUM ACETATE ON FILTRATION RATE[1] IN MAN[2]

Subject		Control	Acetate	Acetate / Control
Age	Sex			
yr.		*cc./min.*	*cc./min.*	
22	F	154	163	1.06
40	M	124	127	1.02
36	M	96.2	95	0.99
9	M	129	130	1.01
29	F	87.8	110	1.25
53	F	60.8	77.5	1.27
10	F	62.3	66.5	1.06

[1] Data on filtration rate are corrected to 1.73 sq. m. body surface area.
[2] For rate of acetate infusion see table 1.

showed an increase in average Tm_{PAH} during acetate infusion ranging from 15 to 44 per cent, while the fourth subject showed no change. Tm_{PAH} was increased by 26 per cent in one of the two patients with essential hypertension, and remained constant in the other. The patient with chronic diffuse glomerulonephritis showed an increase of 43 per cent in the average Tm_{PAH} during acetate infusion. In the five instances in which an increase occurred, the magnitude of the increase appears to be related to the rate of administration of acetate, but no inference can be drawn in this respect because different subjects are under comparison. The infusion of acetate had no marked effect on the filtration rate.

The control values (averages of three urine collection periods) for the C_{PAH} clearance ratio in the three subjects examined were 1.35, 1.29 and [illegible], during acetate infusion this clearance ratio averaged 1.35, 1.25 and 1.17, respectively. The reduction in one subject only cannot be assigned significance; it appears that acetate does not accelerate the tubular excretion of creatinine, a negative effect to be anticipated in view of the current interpretation that acetate accelerates the excretion of compounds which have a R_1H_2COOH group, such as PAH and diodrast.

Since this work was completed McDonald, Shock and Yiengst (8) have reported that the intravenous administration of sodium lactate also increases Tm_{PAH} in man.

Discussion

Mudge and Taggart (9) in the dog and McDonald, Shock and Yiengst (8) in man find only minimal increases in Tm_{PAH} during the infusion of sodium bicarbonate, eliminating an increase in serum pH or bicarbonate content as factors in the effect of acetate and lactate. It is improbable that either compound increases the number of nephrons available for perfusion, since the induction of renal hyperemia in man does not increase diodrast Tm (9).

Our failure to observe the acetate effect in one of the subjects without renal disease and in one hypertensive subject was not related to the rate of acetate administration or the absolute value of Tm_{PAH}. It is possible that this variability in response is a reflection of varying endogenous availability of acetate or related metabolites during Tm_{PAH} measurement. The positive effect of acetate on Tm_{PAH} in the patient with chronic glomerulonephritis presents no unusual feature since this value was not markedly reduced below the normal range.

Summary

The intravenous infusion of sodium acetate (0.08 [illegible] mEq/kg minute) increased Tm_{PAH} by more than 10 per cent of the control value in three of four subjects without renal disease, in one of two subjects with essential hypertension, and in one subject with glomerulonephritis. The exogenous creatinine inulin clearance ratio was not influenced by the infusion of acetate in three subjects without renal disease.

References

1. [illegible] K. J. and J. V. [illegible] *J. Physiol.* [illegible]
2. [illegible] G. H. and J. V. [illegible] *Am. J. Physiol.* [illegible]
3. [illegible] J. V. *Am. J. Med.* [illegible]
4. [illegible] D. and N. [illegible] *Am. J. Physiol.* [illegible]
5. [illegible] W. and H. Chasis *Hypertension and Hypertensive Disease* [illegible]
6. [illegible] H. W., N. [illegible]
7. [illegible] R. W. and H. H. [illegible]
8. [illegible] R. E., N. [illegible]
9. [illegible] H. W., [illegible] H. Chasis, H. A. [illegible]

ITALIAN TRANSLATION

MINIMUM LETHAL DISTANT DOSES AND CAUSES OF DEATH FOLLOWING INTRAVENOUS INJECTION OF SODIUM ACETATE AND NEUTRAL SODIUM TARTRATE SOLUTIONS

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Source: Archivio Italiano di Scienze Famacologich, Vol. II, pp. 495-512, 1933.

I have determined the distant toxicity according to Simon (Archivio di Scienze, Biologiche, Vol. 12, 478, 1928), i.e. the smallest dose of drugs which, after injection into the veins of an animal, does not kill the animal during injection, but sometime after the end of the injection.

It is well known that, in order to achieve this goal, solutions must be injected very slowly so that the rate of injection is never toxic, and that the injection must be slowed down or even stopped for several minutes if toxic phenomena are noted. After the injection was completed, the animal was observed and, if it died, an autopsy was performed, as well as a histological examination of the principal organs fixed in formalin and then stained with hematoxylin and eosin.

I shall now describe the tests.

I. Tests with sodium acetate. The tests were performed on apparently healthy rabbits, kept for several days in the laboratory on a constant diet. The solution of the drug was injected into the right jugular vein at a slow rate (2cc/min), and the injection was temporarily stopped if threatening symptoms appeared.

The aqueous solution of sodium acetate ($CH_3CO_2Na + 3H_2O$) was normal (136.07g/1).

Test no. I--29 February 1932.

Rabbit weighing 1100 g. Into the right jugular vein, 10 cc/kg body weight of Na acetate solution (1.36 g substance) was injected (total amount injected: 11 cc). The duration of the injection was 22 min., and the rabbit remained quiet during the injection. After 60 days, the animal was alive and weighed 1200 g.

Test no. II--29 February 1932.

Rabbit weight: 1400 g. Into the right jugular vein, 15 cc/kg Na acetate solution (2.04 g) was injected (total amount injected: 21 cc). Duration of the injection: 40 min. No disturbances were noted during and after injection. The animal was alive after 60 days and weighed 1470 g, after the weight had dropped considerably in the first few days after injection.

Test no. III--1 March 1932.

Rabbit weight: 1050 g. Into the right jugular vein, 20 cc/kg Na acetate solution (2.72 g) was injected (total amount injected: 21 cc). Duration of injection: 40 min. No disturbances noted during and after injection. In the first days immediately after injection, the rabbit showed a slight weight loss, but the weight increased when the animal was observed later. The animal was alive after 60 days and weighed 1200 g.

Test no IV--1 March 1932.

Rabbit weight: 1000 g. Into the right jugular vein, 24 cc/kg of Na acetate solution (3.34 g) was injected (total amount injected: 24 cc). Duration of injection: 48 min. The animal showed signs of restlessness at the end of injection, and once in a while was shaken by tremors scattered over the entire body, and reacted to touch by trembling. Re-

covered in less than 1 hour, but remained depressed, and this condition lasted for a little more than one day. The animal was alive after 60 days and weighed 1090 g after losing a little weight in the days following the injection.

Test no. V--1 March 1932.

Rabbit weight: 1200 g. Injected into the right jugular vein with 25 cc/kg Na acetate solution (3.4 g). Duration of the injection: 50 min. During injection, the animal was quiet and excreted urine of normal appearance. Nothing else was observed except a slow down of the rate of cardiac pulsations, which, however, later became stronger. After the injection was completed, the animal exhibited a slight spasm in the front body and kept his limbs extended in a lateral direction. Occasionally, the rabbit was shaken by tremors scattered over the whole body and reacted with strong tremors to the slightest touch. After a 1½ hour, the animal had recovered completely, stood up well on his paws and ate, and was merely a little depressed. When observed during the following days, this depression had vanished after 2 days, and the animal appeared to have recovered, but had lost some weight. The rabbit was alive 60 days after injection and weighed 1250 g.

Test no. VI--2 March 1932.

Rabbit weight: 1200 g. Injected into the right jugular vein with 40 cc/kg Na acetate solution (5.44 g). Total amount injected: 48 cc. Duration of the injection: 96 min. After injection of 25 cc, touching the animal caused violent contractions and tremors. The frequency (rate) of the cardiac rhythm was reduced, and breathing was slow. The same symptoms were noted after injection of 35 cc, and in addition, contrac-

tions became spontaneous. The animal passed small amounts of urine, of normal appearance. After being unfastened from the retaining table, the animal did not move and appeared very depressed; it was continously shaken by tremors scattered over the whole body, and this went on for one hour. After two hours, the rabbit lay prone with extended hind limbs and front limbs turned to the right. When these limbs were stretched forward, they returned to the position described above. The animal died 5 hours after injection.

Autopsy findings. Edema of the subcutaneous connective tissue. Opening of the abdomen revealed a bladder highly distended with bloody urine. Microscopic examination showed numerous red globules and granulated urinary cylinders. The liver appeared normal. The kidneys had a normal appearance, the medular portion perhaps slightly reddened. The lungs were slightly congested, of light pink color.

Microscopic findings. Lungs: isolated free epithelia are acted in some alveoles in the alveolar lumen, and some red globules; otherwise, the lungs appear normal. Myocardium: no pathological symptoms. Liver: no pathological symptoms. Kidneys: congestion of glomerular capillaries and of the external medullar portion. The epithelia of the twisted (contorted) tubules appear swollen, as much as their lumen has disappeared.

Test no. VII--4 March 1932.

Rabbit weight: 1400 g. Injected into the right jugular vein with 35 cc/kg Na acetate solution (4.76g). Total amount injected: 49 cc. Duration of injection: 100 min. During injection, the animal, after receiving 29 cc, has 164 cardiac pulsations per minute and 55 breathings per minute. After just being unfastened, the animal has difficulty in standing up on its paws; he attempts to make a few jumps, and seems to

recover, but 3 hours after injection is completed, the muscles of the limbs are extended and rigid, and the animal is occasionally shaken by fibrillary tremors scattered through all muscles. This state of contraction is enhanced as a result of smaller stimuli imparted to the animal, which died 12½ hours after injection.

Autopsy findings. On opening the abdomen, the bladder appears empty and flaccid. Nothing abnormal noted in the liver. In the kidneys, which are edematous, the cortical portion has a normal color. The lungs are greatly congested and have a light pink color. The heart has a systolic contraction.

Microscopic examination. Lungs: in the lumen of some groups of alveoles, numerous red globules and occasionally a subtle, light-blue reticulum are observed. Nothing abnormal in the myocardium. Kindeys: moderate congestion of the capillaries of the medullar portion. The glomerules appear to be flattened and compressed, and only in some points can the capsular space be seen, within which single red globules and some nucleated elements can occasionally be noted. In some areas, the contorted (twisted) tubules have a finely granulated protoplasm, which appears to be irregularly fringed in the portion facing the lumen; in other areas, the epithelia are swollen and the tubular lumen has disappeared. Inside some straight tubules, the presence of coagulated albumin and red globules is noted. Liver: no pathological findings.

Test no VIII--4 March 1932.

Rabbit weight: 1300 g. Injected into the right jugular vein with 27 cc/kg Na acetate solution (3.67 g). Total amount injected: 35.1 cc. Duration of injection: 70 min. During injection, nothing worthy of attention is noted, except that the pulse appears to be stronger and slightly slower. When the animal is freed and placed on the observation table, it attempts to make strolling movements, but is unable to move its limbs, the extensor muscles of

which are in a tonic spasm. At times, the animal is shaken by tremors involving all muscles. After 1 hour, the spasmic state vanishes entirely, and is succeeded by a state of marked depression. On the following day, the animal does not appear to be in a completely normal condition (it moves little, the respiratory rate is diminished, it eats very little). The animal died at night between 5 and 6 a.m., about 30 hours after injection.

Autopsy findings. Heart in systole, lungs colored in part bright-red and in part greyish-red. The liver is rather pale, the kidneys are slightly anemic, except for the external zone of the medular portion, which seems congested.

Microscopic findings. Lungs: almost the entire piece collected shows alveoles filled with a compact, homogeneous, eosin-colored substance and numerous red globules. Nothing abnormal noted in the myocardium. Liver: a vacuolar degeneration is noted in some areas, while all the rest of the hepatic parenchyma appears normal. Kidneys: congestion of the capillaries of the medular portion. The protoplasm of the epithelia in the contorted (twisted) tubules appears to be destroyed in the area facing the lumen of the tubule; the remaining portion has a finely granulated structure. In some points, the nuclei are pale. Inside the straight tubules, a homogeneous, violet-colored substance is sometimes noted, different from that found in scaly epithelia, and also single red globules.

Test no. IX--7 March 1932.

Rabbit weight: 1100 g. Injected into the right jugular vein with 26 cc/kg Na acetate solution (3.54 g). Total amount injected: 28.6 cc. Duration of injection: 60 min. During the injection, nothing worthy of attention takes place. After just being unfastened from the apparatus, the

animal exhibits a slight muscular spasm of the limbs, and at times is shaken by tremors scattered over the entire body and by strong trembling. After 1 hour, these symptoms diminish and finally disappear. The rabbit then falls into a state of depression, manages to stand up on its paws, but refuses to eat. Observed the next day, it appears to be in a condition of intense depression, which becomes more and more pronounced. Does not eat, and shows a reduced breathing activity. The next morning, the animal is found dead in a state of complete rigor mortis. It died about 30 hours after injection.

Autopsy findings. Heart in diastole. Lungs: colored partly wine-red and partly greyish-red. Liver: pale. Kidneys: slightly anemic, except for the external part of the cortical portion, which seems congested. Bladder: moderately distended by a slightly hemorrhagic urine. Microscopic examination revealed the presence of red globules and granular cylinders.

Microscopic examination. Lungs: almost the entire piece collected shows alveoles filled with a compact, homogeneous, eosin-colored substance and numerous red globules. Liver: a vacuolar degeneration is noted in some areas, while the rest of the hepatic parenchyma appears normal. Kidneys: same findings as in the previous test.

II. Tests with neutral sodium tartrate. I have used an aqueous normal solution of neutral sodium tartrate $(CH(OH)COONa)_2 + 2H_2O$, containing 115.029 g of the drug in 1000 cc.

Test no. I--10 March 1932

Rabbit weight: 1120 g. Injected into the right jugular vein with 40 cc/kg of a neutral Na tartrate solution (4.6 g). Total amount injected: 44.2 cc. Duration of injection: 90 min. After 22 cc, the rabbit showed a slow, deep and stertorous breathing; the heart was strong, but the pulse

became slower. After 30 cc, the injection had to be stopped because the rabbit showed signs of suffering and was striken by clonic, shrieking contractions. Cardiac pulsations were 130 per minute. After injection was resumed, the same symptoms were noted, and the pulse rate dropped to 60 beats/min at the end of the injection. Unfastened and placed on the retention table, the rabbit showed stretched and rigid limbs. It tried to assume a normal position, but was unable to do so, and was in a slight opisthotonos (tetanic spasm). After 2 hours, it remained in the same position and also appeared depressed. After noting that the state of depression continued to increase, the rabbit died 5 hours after injection.

Autopsy findings. Bladder empty, lungs congested, with a wine-red color. Heart flaccid, in diastole. Nothing abnormal noted in the liver. Kidneys and medular portion slightly reddened.

Microscopic examination. Lungs: isolated red globules are noted within the lumen of the alveoles; otherwise, the pulmonary tissue appears normal. No pathological findings in the myocardium. Kidneys: glomerules appear congested and in the capsular space of some glomerules a homogeneous substance is noted, present in the form of lumps or as a fine-mesh grid. The protoplasm of the epithelium in twisted (contorted) tubules is partially destroyed, and the remaining part has a granular appearance. A homogeneous, violet-colored substance is noted in the lumen of the collector tubules. Liver: no pathological findings.

Test no. II--11 March 1932

Rabbit weight: 1250 g. Injected into the right jugular vein with 30 cc/kg of neutral Na tartrate solution (3.4 g). Total amount injected: 37.5 cc. Duration of injection: 75 min. During injection, the rabbit exhibits the same symptoms as those described in the preceding test. After being un-

fastened, the animal is unable to rise on its front feet, which lie stretched and rigid on the table; the hind paws, on the contrary, are in a normal position. The animal holds its nose against the table. After 2 hours, is depressed, inert, and from time to time is shaken by tonic-clonic contractions scattered through all its muscles, with a tetanic spasm. The animal died ½ hour after the end of the injection.

Autopsy findings. The bladder is moderately distended with urine, which appears normal. The liver is normal. The medular portion of the kidneys is congested and hyperemic. The heart is in systole and normal. The lungs are reddened and slightly congested.

Microscopic examination. Lungs: isolated red globules are noted inside the lumen of the alveoles, otherwise the pulmonary tissue appears normal. Myocardium: no pathological findings. Kidneys: the glomerules appear congested and a homogeneous substance, present in the form of lumps or as a fine-mesh grid, is noted in the capsular space of some glomerules. The protoplasm of the epithelium of the twisted (contorted) tubules is partially destroyed, and the remaining part has a granular appearance. A homogeneous, violet-colored substance is noted in the lumen of the collector tubules. Liver: no pathological findings.

Test no. III--15 March 1932.

Rabbit weight: 1200 g. Injected into the right jugular vein with 25 cc/kg neutral Na tartrate solution (2.87 g). Total amount injected: 30 cc. Duration of injection: 60 min. After 20 cc, the animal shows signs of restlessness, and rather strong tonic-clonic contractions appear at the end of injection. The cardiac pulsations are slightly slowed down, and the breathing is also slower. One hour after being unfastened, the animal is unable to stand up on its feet, which are extended. Every attempt to place

it in a normal position fails. After 2 hours, it is still in the same position: the nuscles of the entire body appear relaxed, and from time to time generalized tonic-clonic contractions appear, which become more and more frequent. Later, the rear part of the body appears contracted spastically. Upon touching the rabbit lightly, it reacts with fibrillations. The animal died after 20 hours, without any changes in symptoms.

Autopsy findings. The bladder is empty, the liver is normal, as well as the heart, which was arrested in diastole. The lungs are reddened and congested. The kidneys show congestion of the medular portion.

Microscopic examination. Lungs: the alveolar space is filled almost completely with a compact homogeneous substance and isolated red globules. The myocardium is normal. Kidneys: extremely strong congestion of glomerular capillaries. In some areas, the protoplasm of the epithelia of contorted (twisted) tubules is almost destroyed, and only a wide-mesh reticulum remains, enclosing the nucleus which sometimes has disappeared. In other areas, the epithelium is swollen and the protoplasm has a granular appearance, whereas the nucleus appears pale and poorly defined. A compact homogeneous substance and sometimes a few red globules are noted inside the straight tubules and collectors. No pathological findings in the liver.

Test no. IV--15 March 1932.

Rabbit weight: 1050 g. Injected into the right jugular vein with 22.5 cc/kg neutral Na tartrate (2.59 g). Total amount injected: 23.75 cc. Duration of injection: 48 min. Nothing particular noted during injection. One hour after the end of injection the rabbit is unable to assume a normal position, and is the victim of a tonic spasm, which appears more intense on the front limbs than on the hind limbs, and which immobilizes the animal.

It refuses to eat, and remains in this condition until its death, which takes place 24 hours after injection.

Autopsy findings. The bladder is full, the liver has a deep red color, the lungs are reddened here and there, with punctiform hemorrhages. The kidneys are swollen and have a congested medular portion. The heart is normal, in diastole.

Microscopic examination. Lungs: signs of exudation and diapedesis of red globules; in the piece collected, a hemorrhagic focus comprising 8-10 alveoles is noted. Nothing pathological in the myocardium. The liver is normal, and the kidneys show the same signs as in the preceding test.

Test no. V--16 March 1932.

Rabbit weight: 1170 g. Injected into the right jugular vein with 20 cc/kg neutral Na tartrate solution (2.3 g). Total amount injected: 23.4 cc. Duration of injection: 46 min. Nothing particular noted during injection. After being unfastened, the animal at first is unable to stand up on its feet, but after 1½ hr walks well and eats the food offered to it. On the next day, nothing abnormal is noted, except that 36 hours after injection, the animal is struck by extremely violent tonic-clonic contractions and dies during the course of these contractions.

Autopsy findings. The bladder is full, the liver has a deep-red color. The lungs are reddened here and there, with punctiform hemorrhages. The kidneys have a congested medular portion and are swollen. The heart is diastolic and normal.

Microscopic examination. Lungs: signs of exudation and diapedesis in red globules. In the piece collected, a hemorrhagic focus comprising 8-10 alveoles is noted. Nothing pathological in the myocardium and liver. Kidneys: same as in test no. III.

Test no. VI--17 March 1932.

Rabbit weight: 1150 g. Injected into the right jugular vein with 17.5 cc/kg neutral Na tartrate solution (2.01 g). Total amount injected: 20.1 cc. Duration of injection: 40 min. Nothing abnormal noted during injection. After being just unfastened, the animal walks and stands well on its feet. The next day, appears slightly depressed and, when observed 40 hours after injection, is struck by very strong tonic-clonic contractions and dies with extended, rigid limbs.

Autopsy findings. The bladder is full, the liver has a deep-red color. The lungs are reddened here and there, with punctiform hemorrhages. The kidneys have a congested medular portion and are swollen. The heart is diastolic and normal.

Microscopic examination. Lungs: signs of exudation and diapedesis in red globules. A hemorrhagic focus comprising 8-10 alveoles is noted in the piece collected. Nothing pathological in the myocardium and liver. Kidneys: same as in test no. III.

Test no. VII--17 March 1932.

Rabbit weight: 1200 g. Injected into the right jugular vein with 15 cc/kg neutral Na tartrate solution (1.72 g). Total amount injected: 18 cc. Duration of injection: 36 min. Nothing abnormal noted during injection. When unfastened, the animal immediately assumes a normal position and walks well. Observed the next day, appears a little depressed, but eats. The state of depression, however, becomes more and more pronounced, and the rabbit dies after 45 hours, without exhibiting any convulsion symptoms.

Autopsy findings. The bladder is full, the liver has a deep-red color. The lungs are reddened here and there, with punctiform hemorrhages. The

kidneys have a congested medular portion and are swollen. Heart diastolic, normal.

Microscopic examination. Lungs: signs of exudation and diapedesis in red globules. A hemorrhagic focus comprising 8-10 alveoles is noted in the piece collected. Nothing pathological in the myocardium and liver. Kidneys: same as in test no. III.

Test no. VIII--21 March 1932.

Rabbit weight: 1000 g. Injected into the right jugular vein with 12.5 cc/kg neutral Na tartrate solution (1.44 g). Total amount injected: 12.5 cc. Duration of injection: 25 min. No disturbances noted during injection. Observed during the hours of its life, the animal exhibits a state of depression, which gradually increases and culminates in the death of the animal. Breathing becomes more and more rare and superficial, and finally stops 50 hours after injection. The heart continues to beat for several minutes.

Autopsy findings. The bladder is full, the liver has a deep-red color. The lungs are reddened here and there, with punctiform hemorrhages. Kidneys have a congested medular portion and are swollen. Heart diastolic, normal.

Microscopic examination. Lungs: signs of exudation and diapedesis in red globules; a hemorrhagic focus comprising 8-10 alveoles is noted in the piece collected. Myocardium and liver are normal. Kidneys: same as in test no. III.

Test no. IX--21 March 1932.

Rabbit weight: 1100 g. Injected into the right jugular vein with 10 cc/kg neutral Na tartrate solution (1.15 g/kg). Total amount injected: 11 cc. Duration of injection: 22 min. No disturbances noted during injection. During the time the rabbit remains alive, nothing abnormal is

noted, except for a slight state of depression, which grows stronger. During the last 24 hours, the animal eats nothing, and dies after 65 hours; its weight drops by about 100 g, and no signs of excitation were ever observed. During the last 40 min, breathing was very slow and superficial, becoming difficult a few minutes before death, when it stopped. The heart continued to beat for a few minutes.

Autopsy findings. Lungs congested, with punctiform and lenticular hemorrhages. The heart was diastolic, flaccid and normal. The liver was a little darker than normal and moderately friable. The kidneys had a congested and reddened medular portion.

Microscopic examination. Lungs: symptoms of intense congestion and diapedesis of red globules; small hemorrhagic foci, comprising 8-10 alveoles, are noted in the piece collected. Liver: slightly congested; a vacuolar degeneration of the epithelia is noted in some areas. Kidneys: some contorted tubules have a compact and homogeneous appearance, and thus cannot be distinguished either in the nucleus of the epithelium or in the lumen of the tubules; the protoplasm is intensely colored by eosin. In other points, the epithelia coating the contorted tubules have a pallid protoplasm of reticular appearance, and the nucleus is well preserved. Glomerules appear normal. A compact, homogeneous substance is found in the lumen of straight tubules, and the presence of red globules is noted in some of these tubules.

Test no. X--21 March 1932.

Rabbit weight: 1100 g. Injected into the right jugular vein with 5 cc of neutral Na tartrate (0.575 g). Total amount injected: 5.5 cc. Duration of injection: 11 min. No disturbances noted during or after the injection. The wieght drops slightly during the first 20 days. After 60 days, the animal is in good health and has increased it weight to 1190 g.

Test no. XI--21 March 1932.

Rabbit weight: 1400 g. Injected into the right jugular vein with 7.5 cc/kg of neutral Na tartrate solution (0.863 g). Total amount injected: 10.5 cc. Duration of injection: 20 min. No disturbances noted during injection. When observed later, the animal appears listless, eats little. Gradually, it falls into a depressed state, which becomes more pronounced until its death, which takes place 70 hours after injection. Here also, it is noted that the heart continues to beat for sometime after breathing has stopped; breathing became more and more slow, superficial and labored (difficult).

Autopsy findings. Lungs are congested, with punctiform and lenticular hemorrhages. The heart is diastolic and flaccid. The liver is darker than normal and friable. The kidneys have a congested and reddened medular portion.

Microscopic examination. Lungs: signs of intense congestion and diapedesis of red globules; small hemorrhagic foci comprising 8-10 alveoles are noted in the piece collected. Kidneys: same as in test no. IX.

Test no XII--6 April 1932.

Rabbit weight: 1400 g. Injected into right jugular vein with 6 cc/kg neutral Na tartrate solution (0.69 g). Total amount injected: 8.4 cc. Duration of injection: 19 min. Nothing particular was noted during and after injection. The weight of the animal dropped considerably during the first 25 days after injection, but the rabbit later recovered and increased in weight. After 60 days, it was alive and weighed 1500 g.

Test no. XIII--14 April 1932.

Rabbit weight: 1000 g. Injected into the right jugular vein with 6.75 cc/kg neutral Na tartrate solution (0.776 g). No disturbances were noted during injection. The animal was alive after 60 days and wieghted 115 g, after slightly losing some weight in the first 20 days after injection.

III. Examination of the results. We shall now examine the tests for each of the salts used from the following 3 viewpoints:

1) From the standpoint of the minimum lethal dose.

2) From the standpoint of the symptoms observed.

3) From the standpoint of the anatomic-pathological lesions (injuries) noted.

A. Sodium acetate tests

1. From the standpoint of the minimum lethal dose, the following table should be considered.

TABLE 1

1 Num. progres.	2 N. dell'esperienza	3 Peso del coniglio in gr.	4 Acetato di sodio somministrato: cc. di soluz. norm. soluz.	in gr.	in gr. per kg.	5 Esito
1	I	1100	11	1,44	1,30	Vive e sta bene dopo 60 g.
2	II	1400	21	2,81	2,04	" "
3	III	1050	21	2,81	2,72	" "
4	IV	1000	21	3,34	3,34	" "
5	V	1200	30	4,08	3,4	" "
6	IX	1100	26,6	3,88	3,54	" "
7	VIII	1400	35,1	4,52	3,61	Muore dopo h. 36 dall'inie.
8	VII	1400	49	6,66	4,75	" 30 "
9	VI	1200	43	6,93	5,44	" 12,30 "
						" 5 "

1-Case no.
2-Test no.
3-Weight of rabbit, in g.
4-Na acetate administered in g/kg
5-cc of normal solution injected
6-In g/kg
7-Outcome
8-Alive and in good health after 60 days
9-Died 36 hours after injection
10-Died 30 hrs after injection
11-Died 12½ hrs after injection
12-Died 5 hrs after injection

This table shows that rabbits stay alive and in good health up to a dose of 3.4 g/kg, while at a dose of 3.54 g/kg, they die 36 hours after injection. This dose can be considered with sufficient approximation as the minimum lethal distant dose by the intravenous route.

2. In regard to the symptoms observed, I have noted that, at a dose of 1.36 g/kg and up to a dose of 2.7 g/kg, sodium acetate is tolerated quite well by the animals, which did not exhibit any abnormal symptoms whatsoever, either during injection or during the successive period of observation, lasting up to 60 days. All rabbits treated with such doses, after losing weight in the days following the injection, then showed a weight gain ranging from a minimum of 5% to a maximum of 11%.

With doses of 3.34 g/kg and 3.40 g/kg, the animals also tolerated the injection quite well, during which they merely exhibited a slight slowing down of the cardiac rhythm frequency. When the injection was completed, spasms and generalized tremors appeared, which disappeared quite rapidly, however, and were followed (after a little more than 1 hour) by a rather depressive state of slight duration (1-2 days). Two or three days after injection, and after a transitory weight loss, the animals showed a gradual weight increase (of 9% after 60 days).

At doses of 3.54 g/kg and higher doses (2.76, 4.67, 5.44 g/kg) facts are observed which, in my opinion, demonstate a stimulating (exciting) effect of the drug on the nervous system. The rabbits react with tonic-clonic contractions to the stimuli applied, even when these are very light. In addition, in test no VI, where 5.44 g/kg was injected, the rabbit exhibited spontaneous tonic-clonic contractions at the end of the injection.

After completion of the injection, the following symptoms were noted: a spasm of the muscles in the limbs (which were extended and rigid, and reacted with an intensification of this state following mechanical stimuli), interrupted from time to time by spontaneous tonic-clonic contractions, with a tetanic spasm, of brief duration; the spasm resumed after these contractions stopped. These symptoms appeared to be lighter in rabbits which received the minimum lethal dose (3.54 g/kg) or a dose just slightly higher (3.67 g/kg),

in its place, of a general depressive state, which followed a progressively more severe course and was accompanied by a decrease of respiratory activity, which was also gradually more pronounced until the death of the animal took place. In rabbits treated with higher doses (4.67 g/kg and 5.44 g/kg), the symptoms of motor excitation were intense and lasted until death, and breathing was always frequent and superficial.

In all tests, the heart rate underwent a slowdown, which was never very noticeable (significant).

In regard to the results of the anatomic-pathological examination, according to what can be noted from the findings reported, it follows that macro-alterations, and especially microscopic changes are particularly evident in the kidneys, which always have an edematous and congested appearance and which on microscopic examination, exhibit, along with a picture of capillary congestion affecting especially glomerular vessels, more or less extensive areas of vacuolar-granular degeneration of the epithelia of contorted tubules, with a breakdown and disappearance of part of their protoplasm. Such degenerative signs (symptoms) were very intense in those rabbits in which the drug action was more prolonged (tests nos. IX-VIII and VII), whereas 5 hours after injection (test no. VI) only a swelling of the epithelia of contorted tubes was detected. The toxic action of the product is also manifested in the lungs, which always shows the presence in the alveoles of desquamated epithelia and of red globules escaped from the capillaries. In regard to the liver, only signs of vacuolar degeneration are observed in rabbits which survived for a longer itme, whereas in other rabbits nothing pathological has been noted.

From the set of facts reported we can draw the conclusion that the cause of death at high doses can be attributed entirely to the action of the drug on the nervous sytem, whereas with the minimum lethal distant dose,

which allows a longer survival time of the animal, we must also consider lesions (injuries) of the kidneys and lungs, which are incompatible with life.

B. Test with neutral sodium tartrate

These tests must also be considered from 3 standpoints namely:

1) From the standpoint of the minimum lethal dose.

2) From the standpoint of the symptoms observed

3) From the standpoint of macro- and microscopic anatomic-pathological findings.

In regard to the minimum lethal dose, we must consider the following table.

TABLE II

Num. progres.	N. dell'esperienza	Peso del coniglio in gr.	Tartrato neutro di sodio: cc. iniet. di soluz. nor.	in gr.	in gr. per kg.	Esito
1	X	1100	5,5	0,632	0,575	Vive e sta bene dopo 60 g.
2	XII	1400	8,4	0,966	0,69	" "
3	XIII	1000	6,75	0,776	0,776	" "
4	XI	1400	10,5	1,2	0,843	Muore dopo h. 70 dall'inie.
5	IX	1100	11	1,27	1,15	" 65 "
6	VIII	1600	12,5	1,44	1,44	" 60 "
2	VII	1200	18	2,07	1,72	" 45 "
8	VI	1150	20,1	2,32	2,01	" 40 "
9	V	1170	23,4	2,7	2,3	" 36 "
10	IV	1050	23,75	2,73	2,59	" 24 "
11	III	1200	30	3,4	2,87	" 20,15' "
12	II	1250	37,5	4,3	3,4	" 13,30' "
13	I	1120	44,2	5,08	4,6	" 5,30' "

1-Case no.
2-Test no.
3-Rabbit weight, in g.
4-Neutral Na tartrate
5-cc normal solution injected
6-g/kg
7-Outcome
8-Alive and in good health after 60 days
9-Dies 70 hours after injection
10-Dies 65 hours after injection
11-Dies 50 hours after injection
12-Dies 45 hours after injection
13-Dies 40 hours after injection
14-Dies 36 hours after injection
15-Dies 24 hours after injection
16-Dies 20¼ hours after injection

17-Dies 12½ hours after injection
18-Dies 5½ hours after injection

From this table we can see that rabbits stay alive and well up to a dose of 0.776 g/kg, whereas at a dose of 0.863 g/kg they die 70 hours after injection. This dose can be considered with sufficient approximation as the minimum lethal distant dose.

In regard to the symptoms, it can be noted that no symptoms whatsoever are observed with doses lower than the minimym lethal dose (0.863 g/kg). The rabbits were always in good health during and after injection, and gradually put on weight during the 60 days in which they were observed (10%).

With doses of 0.863 to 2.3 g/kg, rabbits did not show any pathological symptoms during injection and the following 24 hours; only after this period, a state of depression set in, which increased steadily and culminated with the death of the animals; however, at doses of 1.91 g/kg (test no. V) and 2.3 g/kg (test no. VI), the animals died from clonic-tonic convulsions which appeared at the last moment. During the first period, breathing was slow and superficial, and the pulse also slowed down. In those rabbits which I was able to observe until the moment of death, it was noted that the heart continued to beat after respiration had stopped.

At doses of 2.59, 2.83, 3.4 and 4.6 g/kg, facts were noted which demonstrate an exciting (stimulating) effect on the nervous sytem. Such facts consist in a hyperexcitability versus external stimuli, in a spastic contraction more or less intense and acattered over the whole body, with the occasional appearance of generalized tonic-clonic contractions. These symptoms were naturally less pronounced (marked) in rabbits which received the lower doses mentioned here, and in which they appeared only sometime after the end of

the injection (test no. IV), whereas in all rabbits treated with higher doses these symptoms were already noted during injection and were particularly intense.

In order to interpret this different action of the tartaric anion according to the various lethal doses administered, we must recall that this anion is a reagent for the calcium ion, which it removes from blood and tissues (L. Sabbatani, Memorie della R. Accademia delle Scienze di Torino, LVI (Serie II), 1904, 459). It is evident, therefore, that only with high doses does such a removal of calcium ions take place as to produce violent convulsions and make life impossible, whereas life is equally not possible with doses which do not produce convulsive phenomena. For this kind of death, we must think of a mechanism different from the action exerted by the tartaric anion, and which is completely unknown to us.

In regard to the results of the macroscopic necrological examination, it follows from the findings reported in the individual tests that the most easily detectable changes are those affecting the lungs, which almost always appeared congested and almost always exhibited small hemorrhagic foci, except in tests nos. I, II, and III. A slight congestion of the liver and kidneys was also observed.

Microscopic examination of the collected pieces of organs showed the presence of alterations in almost all organs examined. These alterations consist as far as lungs are concerned in exudation and diapedesis of red globules, and sometimes in true and actual hemorrhages. In the liver, pathological alterations could be detected only in 2 rabbits which died from smaller doses and which thus survived for a longer time. Such alterations consisted in a vacuolar, more or less diffused and marked degeneration. In the kidneys, along with a glomerular congestion, signs of exudation inside Bowman's capsula

could also be observed. In this organ, however, alterations in the epithelia of tubules were particularly extensive; they consisted in phenomena of vacular-granular degeneration and breakdown of the protoplasm, which were very diffuse and pronounced, and in one case (test no. XI, rabbit which died after 70 hours), a characteristic albuminous degeneration of some tubular segments was noted.

Thus, it is probable that the death of rabbits treated with smaller doses of neutral sodium tartrate can be attributed to a major extent to exudative and hemorrhagic symptoms encountered in the lungs and, to a lesser extent, to renal injuries.

IV. Conclusions

1. The minimum lethal distant dose, by the intravenous route, of sodium acetate in the rabbit is 3.45/kg body weight (equal to 0.0260 gram-equivalent); the corresponding dose for neutral sodium tartrate is 0.863/kg (0.0075 gram-equivalent). Thus, the tartrate is 3 times more toxic than the acetate.

2. With regard to the symptoms observed, it is noted that sodium acetate, given in lethal doses, always produces phenomena of motor excitation. These include spastic symptoms (stretching of the limbs), interrupted from time to time by brief clonic-tonic convulsions; this picture remains unchanged until the death of the animal, when the lethal doses exceed the value mentioned above. With lethal doses below this value, the spastic state is followed by a general depression, whic becomes progressively worse until death.

On the other hand, the following symptoms are noted with sodium tartrate: at very high doses, this salt kills the animal with clonic-tonic convulsions which occur without interruption until death. With lethal doses that are not as high, an initial convulsive period, the duration of which is the shorter the lower the dose, is followed by a progressive period of depression which

leads to death. With the minimum lethal distant dose, but also with slightly higher doses, no period of excitation whatsoever is noted, and the salt exerts a constant depressive effect from the beginning to the end.

3. The cause of death from very high doses of the 2 salts must be attributed to injuries of the central nervous system. With doses producing a slight convulsive period, or with doses which do not exert such an effect, death can in fact be explained as follows: in the case of sodium acetate, it is due primarily to histological injuries (lesions) found in the kidneys and lungs, which by themselves are incompatible with life; in the case of neutral sodium tartrate, it is due to pulmonary exudation and lesions, which lead to the same result.

SUMMARY

Neutral sodium tartrate is 3 times as toxic as sodium acetate. In fact, the minimum lethal distant dose in the rabbit, by the intravenous route, is 0.0075 gram-equivalent for sodium acetate, while the corresponding dose for sodium tartrate is 0.0026 gram-equivalent. Both drugs, at high doses, have a lethal effect as a result of their action on the central nervous system, whereas death caused by the minimum lethal dose is due to a major extent to lesions (injuries) of internal organs (kidneys and lungs).

ISTITUTO DI FARMACOLOGIA SPERIMENTALE DELLA R. UNIVERSITÀ DI PISA
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DOSE MINIMA LETALE LONTANA E CAUSE DELLA MORTE IN SEGUITO AD INIEZIONE ENDOVENOSA DI SOLUZIONI DI ACETATO E TARTRATO NEUTRO DI SODIO

Arch Ital Sci Pharmacol 2: 495-512 (1933)

Ho determinato la tossicità lontana secondo SIMON (1), cioè la più piccola dose dei farmaci che, iniettata nelle vene di un animale, lo uccide non durante l'iniezione, ma qualche tempo dopo la fine di questa.

E' ben noto che per riuscire nello scopo bisogna iniettare le soluzioni con grande lentezza in modo che la velocità non sia mai tossica e rallentare o addirittura sospendere per qualche minuto l'iniezione se si presentano fenomeni tossici. Finita l'iniezione tenevo in osservazione l'animale e, se veniva a morte, praticavo l'autopsia e l'esame istologico dei principali organi, fissati in formalina, colorandoli poi con ematossilina ed eosina.

Passo ora a descrivere le esperienze.

I) ESPERIENZE CON L'ACETATO DI SODIO.

Furono praticate su conigli apparentemente sani, tenuti da qualche giorno in Laboratorio a dieta costante. Iniettai la soluzione del farmaco nella vena giugulare destra con una velocità piccola (due cc. al minuto) ed avevo l'avvertenza di arrestare temporaneamente l'iniezione se apparivano sintomi minacciosi.

La soluzione acquosa di acetato di sodio ($CH_3CO_2Na+3H_2O$) era normale (gr. 136,07 per Litro).

(1) SIMON I. — Archivio di Scienze Biologiche, XII, 1928, 478.

I^a ESPERIENZA — 29 Febbraio 1932.

Coniglio del peso di g. 1100. Gli inietto nella vena giugulare d. cc. 10 della soluzione di acetato di sodio per Kg. (gr. 1,36 di sostanza): complessivamente cc. 11. Durata dell'iniezione 22 minuti. Durante l'iniezione il coniglio si mantiene tranquillo.

E' vivo dopo 60 giorni e pesa gr. 1200.

II^a ESPERIENZA — 29 Febbraio 1932.

Peso del coniglio: gr. 1400. Inietto nella vena giugulare d. cc. 15 della soluzione d'acetato di sodio per Kg. (gr. 2.04 di sostanza): complessivamente cc. 21. Durata dell'iniezione 40'. Nessun disturbo durante e dopo. E' vivo dopo 60 giorni e pesa gr. 1470, dopo che il peso era sensibilmente diminuito nei primi giorni dopo l'iniezione.

III^a ESPERIENZA — 1 Marzo 1932.

Peso del coniglio : gr. 1050. Inietto nella vena giugulare d. cc. 20 per Kg. della soluzione di acetato di sodio (gr. 2,72 di sostanza): complessivamente cc. 21. Durata dell'iniezione 40'. Nessun disturbo durante e dopo. Il coniglio, nei giorni subito dopo l'iniezione, perde leggermente di peso. Però, osservato in seguito, il peso aumenta. E' vivo dopo 60 giorni e pesa 1200 grammi.

IV^a ESPERIENZA — 1 Marzo 1932.

Peso del coniglio gr. 1000. Inietto nella vena giugulare d. cc. 24 per Kg. della soluzione di acetato di sodio (g. 3,34 di sostanza): complessivamente cc. 24. Durata dell'iniezione 48'. Segni di irrequietezza verso la fine dell'iniezione. Ogni tanto è scosso da tremori diffusi a tutto il corpo e, toccandolo, reagisce con sussulti. In meno di un'ora si riprende, ma rimane depresso e tale stato si prolunga poco più di un giorno.

E' vivo dopo 60 giorni e pesa gr. 1090, dopo aver perduto un po' di peso nei giorni successivi all'iniezione.

V^a ESPERIENZA — 1 - 3 - 1932.

Peso del coniglio: gr. 1200. Inietto nella vena giugulare d. cc. 25 per Kg. della soluzione di acetato di sodio (gr. 3,4 di sostanza). Durata dell'iniezione 50'. Durante l'iniezione il coniglio si mantiene tranquillo ed emette urina di aspetto normale; non osservo altro che un rallentamento della frequenza delle pulsazioni cardiache, le quali però diventano più valide. Finita l'iniezione presenta un leggero spasmo del treno anteriore e tiene gli arti estesi lateralmente. Periodicamente è scosso da un tremore diffuso a tutto il corpo e al minimo tocco reagisce con forti sussulti. Dopo un'ora e mezzo l'a-

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nimale si presenta ristabilito completamente, si regge bene sulle zampe e prende cibo: solamente è un po' depresso. Osservato nei giorni successivi, la depressione dopo due giorni sparisce e l'animale sembra ristabilito, però è diminuito di peso. E' vivo dopo 60 giorni dall'iniezione e pesa gr. 1250.

VI^a ESPERIENZA — 2 - 3 - 1932.

Peso del coniglio: gr. 1200. Inietto nella vena giugulare d. cc. 40 per Kg. della soluzione di acetato di sodio (gr. 5,44 di sostanza): complessivamente cc. 48. Durata dell'iniezione 96'. Dopo l'iniezione di 25 cc., toccando l'animale si provocano delle contrazioni violente, dei sussulti. Frequenza del ritmo cardiaco diminuita; anche il respiro è rallentato. Gli stessi fenomeni si notano dopo 35 cc. ed in più le contrazioni si fanno spontanee. L'animale emette scarsa quantità di urina, di aspetto normale. Slegato dal tavolo di contenzione, non si muove, pare molto depresso; è continuamente scosso da tremiti diffusi per tutto il corpo. Così anche dopo un'ora. Dopo due ore il coniglio è in posizione prona con gli arti posteriori estesi, gli anteriori rivolti all'indietro. Portandoli all'innanzi essi ritornano nella posizione descritta.

Muore dopo 5 ore dall'iniezione.

Reperto d'autopsia:

Edema del connettivo sottocutaneo. Aperto l'addome si constata che la vescica è enormemente distesa da urina emorragica. Fatto l'esame microscopico si trovano numerosi globuli rossi e cilindri urinari granulosi. Fegato di aspetto normale. Reni di aspetto normale, la midollare forse un po' arrossata. Polmoni lievemente congesti, di color rosco carico.

Reperto microscopico.

Polmoni: in qualche alveolo si osservano singoli epiteli liberi nel lume alveolare e qualche globulo rosso: del resto il polmone appare normale.

Miocardio: nulla di patologico.

Fegato: nulla di patologico.

Reni: congestione di capillari glomerulari e della parte esterna midollare. Gli epiteli dei tubuli contorti appaiono rigonfi tanto che il loro lume è scomparso.

VII^a ESPERIENZA — 4 - 3 - 1932.

Peso del coniglio: gr. 1400. Inietto nella vena giugulare d. cc. 35 per Kg. della soluzione di acetato di sodio (gr. 4,76 di sostanza): complessivamente cc. 49. Durata dell'iniezione 100'. Durante l'iniezione l'animale, dopo aver ricevuto cc. 29 di soluzione, ha 164 pulsazioni cardiache al minuto e 55 respirazioni. Appena slegato si regge male sulle zampe. Tenta qualche salto e sembra rimettersi, ma dopo tre ore dalla fine dell'iniezione osservo che i muscoli degli arti sono estesi, rigidi e che l'animale di tanto

in tanto viene colto da tremori fibrillari diffusi e tutti i muscoli. Questo stato di contrazione si accentua in seguito agli stimoli più piccoli che vengono portati all'animale. Muore dopo ore 12.30' dall'iniezione.

Reperto d'autopsia:

Aperto l'addome la vescica appare vuota e floscia. Nulla a carico del fegato. Nei reni, che sono edematosi, la corticale è di un colorito normale. I polmoni sono fortemente congesti, di un colore rosco-carico. Il cuore è contratto in sistole.

Esame microscopico:

Polmoni: nel lume di alcun gruppi di alveoli si osservano numerosi globuli rossi e talvolta un sottile reticolo colorato debolmente in azzurro. Nella di anormale nel miocardio.

Reni: modica congestione a carico dei capillari della midollare. I glomeruli appaiono schiacciati e compressi e solo in alcuni punti si apprezza lo spazio capsulare entro al quale si notano talvolta singoli globuli rossi e qualche elemento nucleato. I tubuli contorti hanno in certe zone un protoplasma finemente granuloso, che appare irregolarmente frangiato nella parte rivolta verso il lume; in altre zone gli epitelii sono rigonfi ed il lume del tubulo è scomparso. Nell'interno di alcuni tubuli retti si nota la presenza di albumina coagulata e di globuli rossi.

Fegato: nulla di patologico.

VIII^a ESPERIENZA — 4 - 3 - 1932.

Peso del coniglio gr. 1300. Inietto nella vena giugulare d. cc. 27 per Kg. della soluzione di acetato di sodio (gr. 3,67 di sostanza): complessivamente cc. 35,1. Durata dell'iniezione 70'. Durante l'iniezione non appare nulla di notevole, se si eccettua il fatto che il polso appare più valido e lievemente rallentato. Sciolto l'animale e posto sul tavolo di osservazione, cerca di eseguire movimenti di deambulazione, ma non riesce a muovere gli arti, che hanno i muscoli estensori in spasmo tonico. A tratti è scosso da tremiti generalizzati a tutti i muscoli. Dopo un'ora lo stato di spasmo scompare del tutto e gli subentra uno stato di depressione notevole. Nel giorno seguente l'animale non pare ancora in condizioni del tutto normali (si muove poco, la frequenza respiratoria è diminuita, mangia pochissimo). Muore durante la notte fra il 5 ed il 6, dopo 30 ore circa dall'iniezione.

Reperto d'autopsia:

Cuore in sistole. Polmoni in parte rosso-vivi, in parte rosso-grigiastri. Fegato piuttosto pallido. Reni leggermente anemici, eccetto la zona esterna della midollare, che appare congesta.

Reperto microscopico:

Polmoni: quasi tutto il pezzo prelevato presenta gli alveoli riempiti da una sostanza omogenea compatta colorata dall'eosina e da numerosi globuli rossi. Nulla di anormale nel miocardio.

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Fegato: in alcune zone si nota una degenerazione vacuolare mentre tutto il resto del parenchima epatico appare normale.

Reni: congestione dei capillari della midollare. Il protoplasma degli epitelii dei tubuli contorti appare distrutto nella parte rivolta verso il lume del tubulo; la parte che rimane ha una struttura finemente granulosa. In alcuni punti i nuclei sono pallidi. Nell'interno dei tubuli retti si nota talvolta una sostanza omogenea colorata in violetto, tal'altra degli epiteli sfaldati e singoli globuli rossi.

IX^a ESPERIENZA — 7 - 3 - 1932.

Peso del coniglio: gr. 1100. Inietto nella vena giugulare d. cc. 26 per Kg. della soluzione di acetato di sodio (gr. 3,54 di sostanza): complessivamente cc. 28,6. Durata dell'iniezione 60'. Durante l'iniezione non appare alcun fatto notevole. Appena slegato dall'apparecchio, l'animale presenta un leggero spasmo dei muscoli degli arti. A tratti è scosso da tremiti diffusi a tutto il corpo e da forti sussulti. Dopo un'ora questa sintomatologia si attenua e finisce con lo scomparire. Il coniglio in seguito cade in uno stato di depressione, riesce a reggersi sulle zampe, ma rifiuta il cibo. Osservato il giorno seguente appare sempre in condizioni di intensa depressione, che si fa sempre più accentuata. Non mangia. L'attività respiratoria risulta diminuita.

Il mattino seguente viene trovato morto in completa rigidità cadaverica. Morì circa dopo 30 ore dall'iniezione.

Reperto d'autopsia:

Cuore: in diastole.

Polmoni: in parte di color rosso vinoso ed in parte di colore rosso-grigiastro.

Fegato: pallido.

Reni: leggermente anemici, eccetto nella parte esterna della corticale, che appare congesta.

Vescica: modicamente distesa da un'urina lievemente emorragica. All'esame microscopico si trovano globuli rossi e cilindri granulosi.

Esame microscopico:

Polmoni: quasi tutto il pezzo prelevato presenta gli alveoli riempiti da una sostanza omogenea compatta colorata dall'eosina e da numerosi globuli rossi.

Fegato: in alcune zone si nota una degenerazione vacuolare mentre tutto il resto del parenchima epatico appare normale.

Reni: Come nell'esperienza precedente.

II) ESPERIENZE COL TARTRATO NEUTRO DI SODIO.

Mi sono servito di una soluzione acquosa normale di tartrato neutro di sodio $[CH(OH)COONa]_2 + 2H_2O$, contenente cioè gr. 115,029 del farmaco in 1000 cc.

I^a ESPERIENZA — 10 - 3 - 1932.

Peso del coniglio: gr. 1120. Inietto nella vena giugulare d. cc. 40 per Kg. della soluzione di tartrato neutro di sodio (gr. 4,6 di sostanza): complessivamente cc. 44,2. Durata dell'iniezione 90'. Dopo 22 cc. il coniglio presenta un respiro lento, profondo, stertoroso; il cuore è valido, ma il polso si fa più lento. Dopo 30 cc. si deve sospendere l'iniezione perchè il coniglio dà segni di sofferenza; è colto da contrazioni cloniche, stride. Le pulsazioni cardiache sono 130 al minuto. Riprendendo l'iniezione presenta gli stessi sintomi ed alla fine dell'iniezione la frequenza del polso si abbassa a 60 battiti al minuto. Slegato e messo sul tavolo di contenzione, il coniglio presenta gli arti estesi, rigidi. Cerca di prendere la posizione normale, ma non vi riesce; è in leggero opistotono. Dopo due ore è sempre nella medesima posizione ed insieme si presenta depresso. Constato che lo stato di depressione si accentua sempre di più ed il coniglio muore dopo 5 ore dall'iniezione.

Reperto d'autopsia:

Vescica vuota. Polmoni di colore rosso-vinoso, congesti. Cuore flaccido, in diastole. Nulla a carico del fegato. Reni con midollare un pò arrossata.

Esame microscopico:

Polmoni: nell'interno del lume degli alveoli si notano singoli globuli rossi; del resto, il tessuto polmonare appare normale. Nulla di patologico nel miocardio.

Reni: i glomeruli appaiono congesti e nello spazio capsulare di alcuni di essi si nota una sostanza omogenea disposta a zolle oppure a sottile reticolo. Il protoplasma dell'epitelio dei tubuli contorti è parzialmente distrutto e la parte che rimane ha un aspetto granuloso. Nel lume dei tubuli collettori si nota una sostanza omogenea colorata in violetto.

Fegato: nulla di patologico.

II^a ESPERIENZA — 11 - 3 - 1932.

Peso del coniglio: gr. 1250. Inietto nella giugulare d. cc. 30 per Kg. della soluzione di tartrato neutro di sodio (gr. 3,4 di sostanza): complessivamente cc. 37,5. Durata dell'iniezione 75'. Il coniglio durante l'iniezione presenta gli stessi fenomeni descritti nell'esperienza precedente. Slegato, non può reggersi sulle zampe anteriori, che giacciono estese e rigide sul tavolo; le zampe posteriori invece sono in posizione normale. Tiene il muso appoggia-

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to sul tavolo. Dopo due ore è depresso, inerte, di quando in quando però viene scosso da contrazioni tonico-cloniche diffuse a tutta la muscolatura, con epistotono. Muore dopo mezz'ora dalla fine dell'iniezione.

Reperto d'autopsia:

La vescica è modicamente distesa da urina, apparentemente normale.

Fegato, normale. Reni con midollare congesta ed iperemica. Cuore: in sistole, normale. Polmoni arrossati, lievemente congesti.

Esame microscopico:

Polmoni: nell'interno del lume degli alveoli si notano singoli globuli rossi. Del resto, il tessuto polmonare appare normale. Miocardio: nulla di patologico.

Reni: i glomeruli appaiono congesti e nello spazio capsulare di alcuni di essi si nota una sostanza omogenea disposta a zolle oppure a sottile reticolo. Il protoplasma dell'epitelio dei tubuli contorti è parzialmente distrutto e la parte che rimane ha un aspetto granuloso. Nel lume dei tubuli collettori si nota una sostanza omogenea colorata in violetto.

Fegato: nulla di patologico.

III^a ESPERIENZA — 15 - 3 - 1932.

Peso del coniglio: gr. 1200. Inietto nella vena giugulare d. cc. 25 per Kg. della soluzione di tartrato neutro di sodio (gr. 2,87 di sostanza): complessivamente cc. 30. Durata dell'iniezione 60'. Dopo 20 cc. il coniglio dà segni di irrequietezza e verso la fine dell'iniezione compaiono contrazioni tonico-cloniche abbastanza energiche. Pulsazioni cardiache lievemente rallentate. Respiro anche rallentato. Dopo un'ora da che è slegato, non riesce a reggersi sulle zampe, che sono estese. Ogni tentativo di mettersi in posizione normale è vano. Dopo due ore è sempre nella stessa posizione: la muscolatura di tutto il corpo appare rilasciata e di tanto in tanto compaiono delle contrazioni tonico-cloniche generalizzate, che si fanno sempre più frequenti. In seguito il treno posteriore appare contratto spasticamente. Toccando leggermente il coniglio, reagisce con fibrillazioni. Muore dopo 20 ore senza che la sintomatologia si sia modificata.

Reperto d'autopsia:

La vescica è vuota, il fegato è normale e così pure il cuore, che si è arrestato in diastole. Polmoni arrossati e congesti. Reni con midollare congesta.

Esame microscopico:

Polmoni: lo spazio alveolare è riempito talora quasi completamente da una sostanza omogenea compatta e da singoli globuli rossi. Miocardio normale.

Reni: intensissima congestione dei capillari glomerulari. In alcune zone il protoplasma degli epitelii dei tubuli contorti è quasi distrutto e non rimane che un reticolo a larghe maglie che racchiude il nucleo, che talora è scomparso. In altre zone l'epitelio è rigonfio ed il protoplasma ha un aspetto granuloso, mentre il nucleo appare pallido e mal delimitabile. Nell'interno dei tubuli retti e collettori si nota una sostanza omogenea e compatta e talvolta qualche globulo rosso.

Nulla di patologico nel fegato.

IV^a ESPERIENZA — 15 - 3 - 1932.

Peso del coniglio: gr. 1050. Inietto nella vena giugulare d. cc. 22,5 per Kg. di peso corporeo (gr. 2,59 di sostanza): complessivamente cc. 23,75. Durata dell'iniezione 48'. Nulla di particolare durante l'iniezione. Dopo un'ora dalla fine di questa il coniglio è incapace di mettersi in posizione normale. E' in preda ad uno spasmo tonico, che appare più intenso agli arti anteriori che a quelli posteriori e che li immobilizza. Rifiuta il cibo e in queste condizioni rimane fino alla morte, che avviene 24 ore dopo l'iniezione.

Reperto d'autopsia:

La vescica è piena. Il fegato è di un colorito rosso-cupo; polmoni arrossati qua e là, con emorragie puntiformi. Reni con midollare congesta e tumidi. Cuore in diastole, normale.

Esame microscopico:

Polmoni: fatti di essudazione e di diapedesi di globuli rossi: nel pezzo prelevato si nota un focolaio emorragico che comprende 8-10 alveoli. Nulla di patologico nel miocardio.

Normale il fegato.

Rene come nell'esp. precedente.

V^a ESPERIENZA — 16 - 3 - 1932.

Peso del coniglio: gr. 1170. Inietto nella vena giugulare d. cc. 20 per Kg. della soluzione di tartrato neutro di sodio (gr. 2,3 di sostanza): complessivamente cc. 23,4. Durata dell'iniezione 46'. Nulla di particolare durante l'iniezione. Slegato, l'animale dapprima non riesce a reggersi sulle zampe, ma dopo un'ora e mezza cammina bene e mangia il cibo che gli viene offerto. Il giorno dopo nulla si nota di anormale, se non che dopo 36 ore dall'iniezione il coniglio viene colto da fortissime contrazioni tonico-cloniche e muore durante queste.

Reperto d'autopsia:

La vescica è piena. Il fegato è di un colorito rosso-cupo. Polmoni arrossati qua e là, con emorragie puntiformi. Reni con midollare congesta e tumidi. Cuore in diastole, normale.

Esame microscopico:

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Polmoni: fatti di essudazione e di diapedesi di globuli rossi. Nel pezzo prelevato si nota un focolaio emorragico che comprende 8-10 alveoli.

Nel miocardio nulla di patologico; così nel fegato.

Reni: Come nell'esperienza III.

VI^a ESPERIENZA — 17 - 3 - 1932.

Peso del coniglio: gr. 1150. Inietto nella vena giugulare d. cc. 17,5 della soluzione di tartrato neutro di sodio (gr. 2,01 di sostanza): complessivamente cc. 20,1. Durata dell'iniezione 40'. Nulla di anormale durante l'iniezione. Appena slegato cammina e si regge bene sugli arti. Il giorno dopo appare lievemente depresso e, osservato a 40 ore dall'iniezione, si nota che è preso da fortissime contrazioni tonico-cloniche e muore con gli arti rigidi, estesi.

Reperto d'autopsia:

La vescica è piena. Il fegato è di un colorito rosso cupo. Polmoni arrossati qua e là, emorragie puntiformi. Reni con midollare congesta e tumidi. Cuore in diastole, normale.

Esame microscopico:

Polmoni: fatti di essudazione e di diapedesi di globuli rossi. Nel pezzo prelevato si nota un focolaio emorragico che comprende 8-10 alveoli. Nulla di patologico al miocardio ed al fegato.

Reni: Come nell'esperienza III.

VII^a ESPERIENZA — 17 - 3 - 1932.

Peso del coniglio: 1200. Inietto nella vena giugulare d. cc. 15, per Kg. della soluzione di tartrato neutro di sodio (gr. 1,72 di sostanza): complessivamente cc. 18. Durata dell'iniezione 36'. Nulla di anormale durante l'iniezione. Slegato, prende subito la posizione normale e cammina bene. Osservato il giorno successivo si presenta un po' depresso, però mangia. Lo stato di depressione tuttavia si fa sempre più accentuato ed il coniglio muore dopo 45 ore, senza aver presentato fatti convulsivi.

Reperto d'autopsia:

La vescica è piena. Il fegato è di un colorito rosso cupo. Polmoni arrossati qua e là, emorragie puntiformi. Reni con midollare congesta e tumidi. Cuore in diastole, normale.

Esame microscopico:

Polmoni: fatti di essudazione e di diapedesi di globuli rossi. Nel pezzo prelevato si nota un focolaio emorragico che comprende 8-10 alveoli.

Miocardio e fegato: nulla di patologico.

Rene: come nell'esjerienza III.

VIII^a ESPERIENZA — 21 - 3 - 1932.

Peso del coniglio gr. 1000. Inietto nella vena giugulare d. cc. 12,5 per Kg. della soluzione di tartrato neutro di sodio (gr. 1,44 di sostanza): complessivamente cc. 12,5. Durata dell'iniezione 25'. Nessun disturbo durante l'iniezione. Osservato nelle ore di sopravvivenza, l'animale presentò uno stato di depressione, che andò man mano accentuandosi e che culminò con la morte dell'animale. Il respiro si fece sempre più raro e superficiale; infine cessò dopo 50 ore dall'iniezione. Il cuore continuò a pulsare ancora per qualche minuto.

Reperto d'autopsia:

La vescica è piena. Il fegato è di un colorito rosso-cupo. Polmoni arrossati qua e là, emorragie puntiformi. Reni con midollare congesta e tumidi. Cuore in diastole, normale.

Esame microscopico:

Polmoni: fatti di essudazione e di diapedesi di globuli rossi: nel pezzo prelevato si nota un focolaio emorragico che comprende 8-10 alveoli.

Miocardio e fegato: normali.

Rene: come nell'esperienza III.

IX^a ESPERIENZA — 21 - 3 - 1932.

Peso del coniglio: gr. 1100. Inietto nella vena giugulare d. cc. 10 per Kg. della soluzione di tartrato neutro di sodio (gr. 1,15 di farmaco per Kg.): complessivamente cc. 11. Durata dell'iniezione 22'. Nessun disturbo durante l'iniezione. Il coniglio durante il tempo di sopravvivenza non presentò nulla di anormale, se si eccettua un leggero stato di depressione il quale si fece sempre più intenso.

Nelle ultime 24 ore non mangiò nulla. Morì dopo 65 ore, essendo il peso diminuito di 100 gr. circa e senza aver mai presentato alcun sintoma di eccitazione. Negli ultimi 40 minuti il respiro fu molto lento e superficiale e pochi minuti prima della morte si fece stentato e si arrestò: il cuore pulsò ancora qualche minuto.

Reperto d'autopsia.

Polmoni: congesti, con emorragie puntiformi e lenticolari. Cuore: in diastole, flaccido, normale. Fegato: un pò più scuro della norma e modicamente friabile. Reni: midollare congesta ed arrossata.

Esame microscopico:

Polmoni: fatti di intensa congestione e di diapedesi di globuli rossi; nel pezzo prelevato si notano focolai emorragici che comprendono 8-10 alveoli.

Fegato: leggermente congesto; in alcune zone si nota una degenerazione vacuolare degli epiteli.

Reni: alcuni tubuli contorti hanno un aspetto omogeneo, compatto per

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cui non si distinguono nè il nucleo dell'epitelio, nè il lume dei tubuli, ed il protoplasma è intensamente colorato dall'eosina. In altri punti gli epiteli che rivestono i tubuli contorti hanno un protoplasma pallido con aspetto reticolare ed il nucleo è ben conservato. I glomeruli appaiono normali. Nel lume dei tubuli retti si raccoglie una sostanza omogenea compatta: in alcuni di essi si nota la presenza dei globuli rossi.

X^a ESPERIENZA — 21 - 3 - 1932.

Peso del coniglio gr. 1100. Iniettò nella vena giugulare d. cc. 5 della soluzione di tartrato neutro di sodio (gr. 0,575): complessivamente cc. 5,5. Durata dell'iniezione 11'. Nessun disturbo durante l'iniezione, nè dopo. Il peso diminuisce leggermente nei primi 20 giorni. Dopo 60 giorni l'animale sta bene ed è aumentato di peso: 1190 gr.

XI^a ESPERIENZA — 21 - 3 - 1932.

Peso del coniglio gr. 1400. Iniettò nella vena giugulare cc. 7,5 per Kg. della soluzione di tartrato neutro di sodio (gr. 0,863 di sostanza): complessivamente cc. 10,5. Durata dell'iniezione 20'. Nessun disturbo durante l'iniezione. Osservato dopo l'animale presentasi svogliato, mangia poco. A poco a poco viene preso da uno stato di depressione, che va accentuandosi sempre più fino alla sua morte, la quale avviene dopo 70 ore dall'iniezione. Anche qui si osservò che il cuore continuò a battere per qualche tempo dopo che il respiro era cessato; il respiro si era fatto sempre più lento ed era divenuto superficiale e stentato.

Reperto d'autopsia.

Polmoni: congesti, con emorragie puntiformi e lenticolari.

Cuore: in diastole, flaccido.

Fegato: più scuro della norma e friabile.

Reni: midollare congesta ed arrossata.

Esame microscopico:

Polmoni: fatti di intensa congestione e di diapedesi di globuli rossi: nel pezzo prelevato si notano focolai emorragici che comprendono 8-10 alveoli.

Reni: come nell'esperienza IX.

XII^a ESPERIENZA — 6 - 4 - 1932.

Peso del coniglio gr. 1400. Iniettò nella vena giugulare d. cc. 6 per Kg. della soluzione di tartrato neutro di sodio (gr. 0,69 di sostanza): complessivamente cc. 8,4. Durata dell'iniezione 19'. Nulla di particolare durante l'iniezione e dopo. Il peso dell'animale si fa sensibilmente minore nei primi 25 giorni dopo l'iniezione, ma poi il coniglio si riprende ed aumenta di peso. Vive dopo 60 giorni e pesa gr. 1500.

XIII^a ESPERIENZA — 14 - 4 - 1932.

Peso del coniglio gr. 1000. Iniettò nella vena giugulare d. cc. 6,75 per Kg. di soluzione di tartrato neutro di sodio (gr. 0,776). Nessun disturbo durante l'iniezione. Vive dopo 60 giorni e pesa gr. 1150, dopo che il peso era un pò diminuito nei 20 giorni successivi all'iniezione.

III) Esame dei risultati ottenuti.

Conviene ora esaminare le esperienze per ognuno dei sali usati, da tre punti di vista.

1° - Da quello della dose minima letale;

2° - Da quello della sintomatologia;

3° - Da quello delle lesioni anatomo-patologiche.

A) Esperienze con acetato di sodio:

1° - Dal punto di vista della dose minima letale si consideri la seguente tabella:

TABELLA I^a

Num. progres.	N. dell'esperienza	Peso del coniglio in gr.	Acetato di sodio somministrato			Esito
			cc. di soluz. norm. iniet.	in gr.	in gr. per kg.	
1	I	1100	11	1,44	1,36	Vive e sta bene dopo 60 g.
2	II	1400	21	2,84	2,04	" "
3	III	1050	21	2,84	2,72	" "
4	IV	1000	24	3,34	3,34	" "
5	V	1200	30	4,08	3,4	" "
6	IX	1100	28,6	3,88	3,54	Muore dopo h. 36 dell'inie.
7	VIII	1300	35,1	4,52	3,67	" 30 "
8	VII	1400	49	6,66	4,76	" 12,30 "
9	VI	1200	48	6,53	5,44	" 5 "

Da essa risulta che i conigli fino alla dose di gr. 3,4 per Kg. vivono e stanno bene, mentre con la dose di gr. 3,54 muoiono a 36 ore di distanza dall'iniezione. Questa dose si può ritenere con sufficiente approssimazione la minima letale lontana per via endovenosa.

2° - Per quanto riguarda la sintomatologia ho rilevato: che con la dose di gr. 1,36 fino alla dose di gr. 2,7 per Kg. l'acetato di sodio è stato tollerato benissimo dagli animali, i quali non hanno presentato alcun che di anormale, nè durante l'iniezione nè durante il successivo periodo di osservazione, che si prolungò per

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60 giorni. Tutti i conigli trattati con queste dosi, dopo una perdita nei giorni successivi all'iniezione, poi crebbero di peso da un minimo del 5 % ad un massimo dell'11 %.

Con le dosi di gr. 3,34 e 3,40 per Kg. sopportarono pure molto bene l'iniezione, durante la quale ebbero a subire soltanto un leggero rallentamento della frequenza del ritmo cardiaco. Finita l'iniezione apparvero spasmi, tremori generalizzati che scomparvero però ben presto ed ai quali seguiva (dopo poco più di un'ora) uno stato piuttosto depressivo di lieve durata (1-2 giorni). Due. tre giorni dopo l'iniezione gli animali apparivano completamente normali e, dopo una diminuzione transitoria, crebbero in seguito progressivamente di peso (9 % dopo 60 giorni).

Con la dose di gr. 3,54 per Kg. e con quelle superiori (3,76 - 4,67 - 5,44) si rilevarono fatti che, a mio avviso, dimostrano un'azione eccitante del farmaco sul sistema nervoso. I conigli reagivano con contrazioni tonico-cloniche agli stimoli che venivano loro apportati, anche se questi erano molto leggeri. Nell'esperienza VI, inoltre, nella quale iniettai gr. 5,44 per Kg., il coniglio manifestò sul finire dell'iniezione contrazioni tonico-cloniche spontanee.

Ad iniezione finita si osservarono: spasmo della muscolatura degli arti (che erano estesi e rigidi e reagivano con un'intensificazione di questo stato in seguito a stimoli meccanici) interrotto di quando in quando da contrazioni tonico-loniche spontanee, con opistotono, di breve durata, cessate le quali lo spasmo riprendeva. Questa sintomatologia apparve assai più leggera nei conigli che avevano avuto la dose minima letale (gr. 3,54 per Kg.) o quella immediatamente più elevata (gr. 3,67 per Kg.) nei quali, inoltre, dopo alcune ore, si osservò un'attenuazione progressiva dello spasmo e lo stabilirsi, al suo posto, di uno stato depressivo generale, che andò facendosi sempre più grave ed era accompagnato da una diminuzione dell'attività respiratoria, anch'essa progressivamente accentuantesi fino alla morte dell'animale. Nei conigli trattati con le dosi più elevate (gr. 4,67 e 5,44 per Kg.) i fenomeni di eccitazione motoria durarono intensi fino alla morte ed il respiro apparve sempre frequente e superficiale.

In tutte le esperienze la frequenza del cuore subì un rallentamento che non fu mai molto notevole.

Per quanto riguarda i risultati dell'esame anatomo-patologico, secondo quello che si può rilevare dai reperti riferiti, risulta che le alterazioni macro e specialmente quelle microscopiche riguardano particolarmente il rene, che si presentò sempre congesto ed edematoso e che all'esame microscopico rivelò, accanto ad un quadro di congestione capillare a carico specialmente dei vasi glomerulari, delle zone più o meno estese di degenerazione vacuolo-granulosa degli epitelî dei tubuli contorti, con disgregazione e scomparsa di una parte del loro protoplasma. Tali fatti degenerativi furono molto intensi in quei conigli nei quali il farmaco agì più a lungo (esp. IX - VIII e VII) mentre 5 ore dopo l'iniezione (esp. VI) si rilevò solamente un rigonfiamento degli epitelî dei tubuli contorti. L'azione tossica della sostanza si manifestò anche sul polmone, per quanto almeno si può dedurre dall'esame microscopico, che denunciò sempre la presenza negli alveoli di epitelî desquamati e di globuli rossi fuorusciti dai capillari. Nei riguardi del fegato si osservarono soltanto dei segni di degenerazione vacuolare nel coniglio sopravvissuto più a lungo, mentre negli altri non si notò mai nulla di patologico.

* * *

Dal complesso dei fatti riferiti si può trarre la conclusione che la causa della morte con alte dosi si deve attribuire interamente all'azione del farmaco sul sistema nervoso mentre con la dose minima letale lontana, la quale permette una sopravvivenza maggiore dell'animale, bisogna tenere conto anche delle lesioni del rene e del polmone, incompatibili con la vita.

B) Esperienze con tartrato neutro di sodio.

Anche queste esperienze bisogna considerarle da tre punti di vista:

1° della dose minima letale;

2° della sintomatologia;

3° del reperto anatomo-patologico macro- e microscopico.

Per quanto riguarda la dose minima letale si consideri la seguente tabella:

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TABELLA II^a

Num. progres.	N. dell'esperienza	Peso del coniglio in gr.	Tartrato neutro di sodio			Esito
			cc. iniet. di soluz. nor.	in gr.	in gr. per kg.	
1	X	1100	5,5	0,632	0,575	Vive e sta bene dopo 60 g.
2	XII	1400	8,4	0,966	0,69	" "
3	XIII	1000	6,75	0,776	0,776	" "
4	XI	1400	10,5	1,2	0,863	Muore dopo h. 70 dall'inie.
5	IX	1100	11	1,27	1,15	" 65 "
6	VIII	1000	12,5	1,44	1,44	" 50 "
2	VII	1200	18	2,07	1,72	" 45 "
8	VI	1150	20.1	2,32	2,01	" 40 "
9	V	1170	23,4	2,7	2.3	" 36 "
10	IV	1050	23,75	2,73	2.59	" 24 "
11	III	1200	30	3,4	2.87	" 20,15' "
12	II	1250	37,5	4,3	3,4	" 12,30' "
13	I	1120	44,2	5,08	4.6	" 5,30' "

Da essa risulta che i conigli fino alla dose di gr. 0,776 per Kg., vivono e stanno bene mentre con la dose di gr. 0,863 muoiono a 70 ore di distanza dalla iniezione. Si può ritenere con sufficiente approssimazione che questa dose sia la minima letale lontana.

Per quanto riguarda la sintomatologia c'è da rilevare che essa fu completamente muta con le dosi inferiori alla minima letale (gr. 0,863 per Kg.). I conigli stettero sempre bene sia durante che dopo l'iniezione e crebbero progressivamente di peso nei 60 giorni durante i quali stettero in osservazione (10 %).

Con le dosi da gr. 0,863 fino a 2.3 i conigli nulla di patologico mostrarono durante l'iniezione e nelle 24 ore successive; soltanto dopo questo periodo si stabilì uno stato di depressione che si fece sempre più intenso e culminò con la morte dell'animale che però con le dosi di gr. 1,91 (esp. V) e 2,3 per Kg. (esp. VI) morì fra convulsioni clonico-toniche sopravvenute all'ultimo momento. Durante il primo periodo il respiro si mantenne lento e superficiale ed il polso ebbe pure a subire un rallentamento. Nei conigli che ho potuto seguire fino al momento della morte ho osservato che il cuore continuava a pulsare dopo l'arresto del respiro.

Con le dosi di gr. 2,59 - 2,83 - 3,4 - 4,6 comparvero fatti che testimoniavano un'azione eccitante sul sistema nervoso. Tali fatti consistettero in una ipereccitabilità di fronte agli stimoli esterni, in contrazione spastica più o meno intensa e diffusa a tutto il corpo, con comparsa di quando in quando di contrazioni tonico-cloniche generalizzate. Questa sintomatologia era naturalmente meno

accentuata nei conigli che ricevettero le dosi più basse ora menzionate e nei quali comparve soltanto qualche tempo dopo la fine dell'iniezione (esp. IV), mentre in tutti quelli trattati con dosi più alte comparve già durante l'iniezione e fu più particolarmente intensa.

Per interpretare questa differente azione dell'anione tartarico a seconda delle diverse dosi letali somministrate si ricordi che questo anione è un reattivo dell'jone calcio, che sottrae al sangue ed ai tessuti (1). E' evidente però che solamente con le dosi alte avviene una sottrazione del calcio jone tale da produrre convulsioni violente e da rendere impossibile la vita, mentre questa è ugualmente non possibile con dosi le quali non dànno una fenomenologia convulsiva. Per questo genere di morte bisogna pensare ad un meccanismo differente di azione dell'anione tartarico, a noi completamente ignoto.

Circa i risultati dell'esame necrologico macroscopico, dai referti riportati nelle singole esperienze risulta che le alterazioni più facilmente rilevabili furono quelle a carico dei polmoni, che apparvero quasi sempre congesti e presentarono quasi sempre dei focolai emorragici, eccetto che nelle esperienze I, II, III. Un leggero stato di congestione fu osservato anche a carico del fegato e del rene.

L'esame microscopico dei pezzi prelevati mise in evidenza alterazioni di quasi tutti gli organi studiati. Queste alterazioni consistettero, per quanto riguarda i polmoni, in fatti di essudazione con diapedesi di globuli rossi e talvolta in vere e proprie emorragie. Nel fegato si poterono constatare delle alterazioni patologiche soltanto nei due conigli venuti a morte con le dosi più piccole e che sopravvissero quindi per un tempo più lungo. Tali alterazioni consistettero in una degenerazione vacuolare più o meno diffusa e pronunciata. Nei reni ho potuto osservare talvolta, accanto ad una congestione glomerulare, anche dei fatti di essudazione nell'interno della capsula del Bowmann. Per questo organo però furono particolarmente intense le alterazioni a carico degli epiteli dei tu-

(1) Sabbatani L. — Memorie della R. Accademia delle Scienze di Torino, LIV (Serie II), 1904, 459.

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buli. Esse consistettero in fenomeni di degenerazione vacuolo-granulosa e di disgregazione del protoplasma, molto diffuse ed accentuate, ed in un caso (esp. XI coniglio, morto dopo 70 ore) si potè osservare una caratteristica degenerazione albuminosa di alcuni tratti dei tubuli.

E' probabile perciò che la morte dei conigli trattati con le dosi più piccole di tartrato neutro di sodio si possa attribuire in massima parte ai fatti essudativi ed emorragici riscontrati nei polmoni ed, in grado assai minore, alle lesioni renali.

IV) Conclusioni.

1° - La dose minima letale lontana per via endovenosa di acetato di sodio nel coniglio è di gr. 3,45 (= gr. eq. 0,0260) per Kg. corporeo; la dose corrispondente di tartrato neutro di sodio è di gr. 0,863 (gr eq. 0,0075). Quindi il tartrato è tossico tre volte più dell'acetato.

2° - Per quanto riguarda la sintomatologia si osserva che l'acetato di sodio con le dosi mortali produce sempre fenomeni di eccitazione motoria. Questi sono spastici (estensione degli arti), interrotti ogni tanto da brevi convulsioni clonico-toniche ed il quadro dura immodificato fino alla morte dell'animale quando la dose letale oltrepassi un dato valore; con le dosi letali inferiori a tale valore allo stato spastico subentra una depressione generale, che si aggrava progressivamente fino alla morte.

Il tartrato di sodio invece dà la seguente sintomatologia: con dosi molto elevate uccide con convulsioni clonico-toniche che si succedono ininterrottamente fino alla morte; con dosi letali meno elevate ad un primo periodo convulsivo, il quale dura tanto meno quanto più bassa è la dose, subentra un periodo di depressione progressiva che conduce a morte; con la dose minima letale lontana, ma anche con dosi un po' superiori, non si osserva alcun periodo di eccitazione e l'azione è dal principio alla fine costantemente depressiva.

3° - La causa della morte con dosi molto elevate dei due farmaci si deve attribuire a lesioni del sistema nervoso centrale. Con le dosi che dànno un lieve periodo convulsivo o che non lo dànno affatto la morte si può spiegare: nel caso dell'acetato in massima

parte con le lesioni istologiche riscontrate nel rene e nel polmone, per sè stesse incompatibili con la vita; nel caso del tartrato neutro di sodio con l'essudato e le lesioni polmonari, che portano allo stesso risultato.

RIASSUNTO

Il tartrato neutro di sodio è tre volte più tossico dell'acetato sodico. Infatti la dose minima mortale a distanza per via endovenosa è di gr. eq. 0,0075 per chilo di coniglio per l'acetato, mentre la dose corrispondente di tartrato è di g. eq. 0,0026. Ambedue i farmaci in dose elevata uccidono per azione sul sistema nervoso centrale, mentre per la dose minima mortale la morte è dovuta per la massima parte a delle lesioni di organi interni (reni e polmoni).

RÉSUMÉ

Le Tartrate neutre de sodium est outre trois fois plus toxique de l'acetate de sodium. En effet la dose minime mortelle à distance par voie intraveineuse est de g. eq. 0,0075 par Kgr. de lapine pour l'acétate, tandis que la dose correspondante est de g. eq. 0,0026 pour le tartrate. Tous les deux médicaments en doses élevées tuent par une action sur le système nerveux central, tandis que par la dose minime mortelle la mort est dûe par la plupart à de lésions du reins et du poumons.

ZUSAMMENFASSUNG

Das neutrale Sodiumtartrat ist dreimal mehr giftig als Natriumacetat. Die minimal fernhin tödlich wirkende Dosis durch Veneneinspritzung ist nämlich für das Natriumacetat gr. eq. 0,0075 pro kilo beim Kaninchen, während die entsprechende Dosis von Natriumtartrat ist gr. eq. 0,0026. Beide Arzneien in starken Dosen töten durch ihre Wirkung auch dàns Central-nervensystem, während für die kleine minimal tödlichen Dosen der Tod erfolgt grösstenteils durch Verletzungen der inneren Organen (Niere und Lungen).

SUMMARY

The neutral sodium tartrate is threfold more poisonous as the sodium acetate. For the acetate the minimal lethal dose, when intravenously injected, is in the rabbit of eq. 0,0075 pro Kilogram, for the tartrate of eq. gr. 0,0026. Both the drugs in high doses Kill in the animal because of a direct action on the central nervous system. During minimal lethal doses the dead is due to alterations of internal organs (Kidneys and Lungs).

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Physiological Action of Acetic Acid in Living Tissues

STARTING from the general thesis that volatile acids are produced in large quantity in the rumen of the sheep, McAnally and Phillipson[1] showed that some at all events of these acids are absorbed from the rumen and are found in considerably greater quantities in the blood coming from that organ and from the large intestine than in the blood coming from other parts of the alimentary canal.

They showed further that of the acids so absorbed acetic appears to be the preponderant one.

The fate of acetic acid then demands consideration. Among other possibilities is that of its being destroyed in the tissues. To test this the mammalian (rabbit's) heart was perfused with Locke's solution in which, for the glucose, sodium acetate was substituted.

As the accompanying table shows, the sodium acetate disappeared on the same sort of scale as Locke and Rosenheim[2] found glucose to be consumed.

Serial number	Weight of heart (gm.)	2-hour period	Acetate introduced at the commencement of each 2-hour period (mgm.)	Acetate disappearing in 2-hour period (mgm.)	Remarks
1	2·8	1st period	134	56	
		2nd ,,	,,	52	
		3rd ,,	,,	28	
2	5·35	1st ,,		28	Heart not beating
3	4·60	1st ,,	,,	66	
		2nd ,,	,,	54	
4	5·2	1st ,,	,,	36	1·7 mgm. acetate recovered from heart at end of exp.
		2nd ,,	,,	34	
5	4·6	1st ,,	141	54	0·5 ,, ,,
Controls in which the heart was replaced by a piece of rubber tubing.					
1	—	—	134	5	
2	—	—	134	4	

That acetate has some merit, however, under the conditions of our experiments, is shown by the fact that, whereas we have maintained the hearts beating up to 4–6 hours on solution containing acetate, on the same solutions, free of acetate, they have not beat for more than two hours.

Whether or no the acetic acid is oxidized is under investigation, but whatever its fate may be, the fact that it does disappear opens wide fields for speculation.

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Feb. 24.

[1] McAnally, R., and Phillipson, H. T., *Proc. Physiol. Soc.*; *J. Physiol.* (in the press).
[2] Locke, F. S., and Rosenheim, O., *J. Physiol.*, 36, 205 (1907).

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TRANSFER OF SHORT-CHAIN FATTY ACIDS BY THE INTESTINE

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

METHODS

In vitro. The preparation was the sac of everted small intestine of the rat (Wilson & Wiseman, 1954). The sacs were 30 cm long, made from the jejunum of the rat, contained 3 ml. bicarbonate saline (Krebs & Henseleit, 1932) with 500–1000 mg glucose/100 ml., and were suspended in 45–50 ml. of the same solution. They were shaken at a rate of 80 c/min for 60 min at 38° C in conical flasks, as described by Parsons, Smyth & Taylor (1958). Two types of experiments were done as also described by these authors: (1) transfer experiments, in which the object of the experiment was to measure the changes in the mucosal and serosal fluids; and (2) metabolism experiments, where the object was to estimate the total amount of fatty acid disappearing during the course of the experiment. In the metabolism experiments, the method was modified as follows. After incubation of the sac for 1 hr the flask was immersed in an ice–salt freezing mixture (approximately −10° C) until the contents froze (approximately 3 min). Cold water was then run over the outside of the flask until the contents just thawed. The sac was removed and ground with 1 ml. of acid-washed sand, 5 ml. 7% (w/v) $CuSO_4.5H_2O$ and 5 ml. 10% (w/v) $Na_2WO_4.2H_2O$. The ground-up tissue together with the deproteinizing reagents was pooled with the flask contents, and the total volume made up to 100 ml. After filtering, samples were taken for estimation of volatile fatty acid.

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

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

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

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

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

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

RESULTS

Metabolism of fatty acids

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

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

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

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

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

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

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

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

Transfer of fatty acids

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

Significance of serosal transfer

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

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

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

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

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

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

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

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

Effect of variations in fatty acid concentration

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

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

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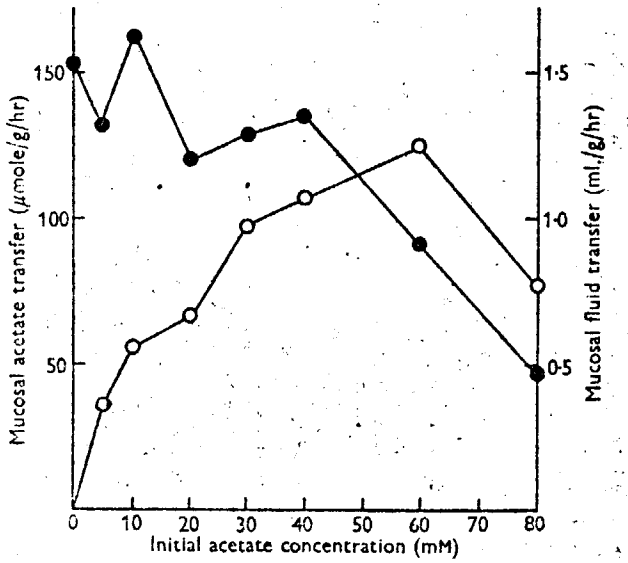

Fig. 1. Effect of initial acetate concentration on mucosal fluid transfer (●) and mucosal acetate transfer (○).

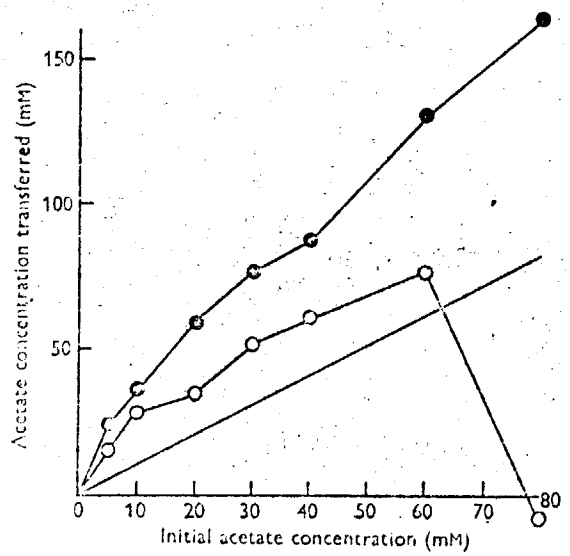

Fig. 2. Effect of initial acetate concentration on the mucosal acetate concentration transferred (●) and serosal acetate concentration transferred (○). The straight line through the origin indicates what would happen if the concentration transferred was equal to the initial concentration.

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

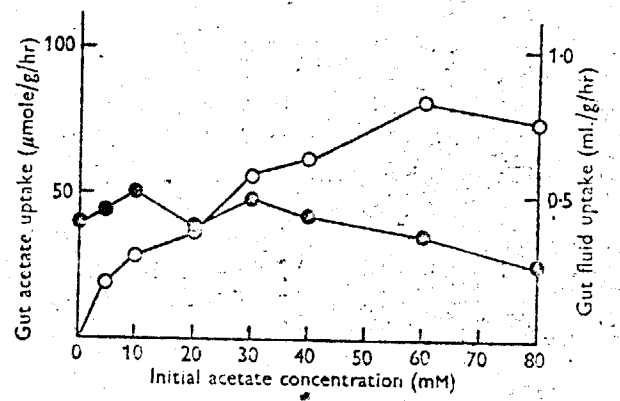

Fig. 3. Effect of initial acetate concentration on gut fluid uptake (●) and gut acetate uptake (○).

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

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

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

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

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

Michaelis–Menten kinetics

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

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

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

Transfer of other fatty acids

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

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

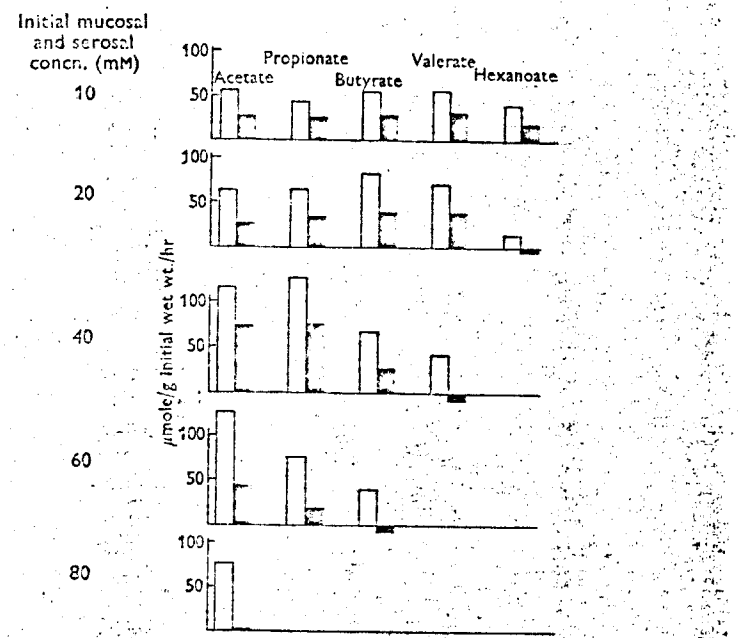

Fig. 4. Relation between length of carbon chain and transfer of fatty acid at various concentrations. The mucosal transfer is shown by white rectangles and the serosal transfer by black rectangles (μmole/g initial wet wt./hr). The lengths of carbon chain were 2 (acetate), 3 (propionate), 4 (butyrate),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

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

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

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

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

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

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

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

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

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

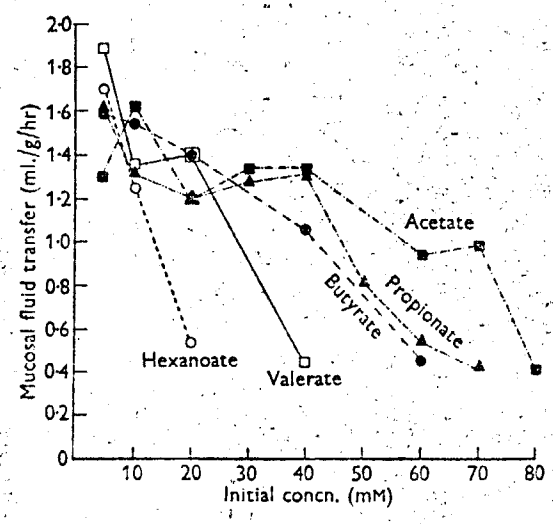

Fig. 5. Effect of initial concentration of a number of fatty acids on the mucosal fluid transfer.

Experiments in vivo

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

SUMMARY

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

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

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

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

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

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REFERENCES

Agar, W. T., Hird, F. J. R. & Sidhu, G. S. (1953). The active absorption of amino-acids by the intestine. *J. Physiol.* **121**, 255–263.

Brodie, B. B. & Hogben, C. A. M. (1957). Some physico-chemical factors in drug action. *J. Pharm.* **9**, 345–380.

Crane, R. K. & Wilson, T. H. (1958). In vitro method for the study of the rate of intestinal absorption of sugars. *J. appl. Physiol.* **12**, 145–146.

Darlington, W. A. & Quastel, J. H. (1953). Absorption of sugars from isolated surviving intestine. *Arch. Biochem.* **43**, 194–207.

Davies, H. & Jowett, G. H. (1959). Statistical appendix to Jervis, E. L. & Smyth, D. H. (1959). The effect of concentrations of amino acids on their rate of absorption from the intestine. *J. Physiol.* **149**, 433–441.

Deuel, H. J., Hallman, L. & Reifman, A. (1941). The rate of absorption of various fatty acids by the rat. *J. Nutr.* **21**, 373–382.

Fisher, R. B. & Parsons, D. S. (1953*a*). Galactose absorption from the surviving small intestine of the rat. *J. Physiol.* **119**, 224–232.

Fisher, R. B. & Parsons, D. S. (1953*b*). Glucose movements across the wall of the rat small intestine. *J. Physiol.* **119**, 210–223.

Jervis, E. L., Johnson, F. R. Sheff, M. F. & Smyth, D. H. (1956). The effect of phlorhizin on intestinal absorption and intestinal phosphatase. *J. Physiol.* **134**, 675–688.

Jervis, E. L. & Smyth, D. H. (1959*a*). Competition between enantiomorphs of amino acids during intestinal absorption. *J. Physiol.* **145**, 57–65.

Jervis, E. L. & Smyth, D. H. (1959*b*). The effect of concentrations of amino acids on their rate of absorption from the intestine. *J. Physiol.* **149**, 433–441.

Jervis, E. L. & Smyth, D. H. (1960). The active transfer of D-methionine by the rat intestine *in vitro*. *J. Physiol.* **151**, 51–58.

Krebs, H. A. & Henseleit, K. (1932). Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe-Seyl. Z.* **210**, 33–66.

Lineweaver, H. & Burk, D. (1934). The determination of enzyme dissociation constants. *J. Amer. chem. Soc.* **56**, 658–666.

Michaelis, L. & Menten, M. L. (1913). Die Kinetik der Invertinwirkung. *Biochem. Z.* **49**, 333–369.

Parsons, B. J., Smyth, D. H. & Taylor, C. B. (1958). The action of phlorrhizin on the intestinal transfer of glucose and water *in vitro*. *J. Physiol.* **144**, 387–402.

Riklis, E. & Quastel, J. H. (1958). Effects of cations on sugar absorption by isolated surviving guinea pig intestine. *Can. J. Biochem. Physiol.* **36**, 347–362.

Sheff, M. F. & Smyth, D. H. (1955). An apparatus for the study of *in vivo* intestinal absorption in the rat. *J. Physiol.* **128**, 67*P*.

Smyth, D. H. & Taylor, C. B. (1957). Transfer of water and solutes by an *in vitro* intestinal preparation. *J. Physiol.* **136**, 632–648.

Smyth, D. H. & Taylor, C. B. (1958). Intestinal transfer of short-chain fatty acids *in vitro*. *J. Physiol.* **141**, 73–80.

Widdas, W. F. (1952). Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer. *J. Physiol.* **118**, 23–39.

Wilson, T. H. (1954). Concentration gradients of lactate, hydrogen and some other ions across the intestine *in vitro*. *Biochem. J.* **56**, 521–527.

Wilson, T. H. (1956*a*). Fluid movement across the wall of the small intestine *in vitro*. *Amer. J. Physiol.* **187**, 244–246.

Wilson, T. H. (1956*b*). The role of lactic acid production in glucose absorption from the intestine. *J. biol. Chem.* **222**, 751–763.

Wilson, T. H. (1956*c*). A modified method for study of intestinal absorption in vitro. *J. appl. Physiol.* **9**, 137–140.

Wilson, T. H. & Wiseman, G. (1954). The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. *J. Physiol.* **123**, 116–125.

Wiseman, G. (1955). Preferential transference of amino-acids from amino-acid mixtures by sacs of everted small intestine of the golden hamster (*Mesocricetus auratus*). *J. Physiol.* [illegible]

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19, No. 6, 1965 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

CONCERNING THE FORM IN WHICH ACETYL UNITS PRODUCED IN MITOCHONDRIA ARE TRANSFERRED TO THE SITE OF DE NOVO FATTY ACID SYNTHESIS IN THE CELL*

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It has been demonstrated that enzymes involved in conversion of glucose-6-phosphate to pyruvate (1) and in de novo‡ formation of fatty acids (2-5) are located mainly in cell sap, whereas those concerned with conversion of pyruvate to acetyl-CoA are in the mitochondria (1,6). This raises the interesting question of how acetyl-CoA, or a derivative of it, is transported from the mitochondria to the site in the cell where de novo fatty acid synthesis takes place. Four possibilities have been suggested (7,8): (a) Acetyl-CoA diffuses as such across the mitochondrial membrane (9); (b) Acetyl-CoA is first hydrolyzed in the mitochondria, and the acetate formed there is then transported to cell sap where it is reconverted to acetyl-CoA (10); (c) Acetyl-CoA is converted by mitochondrial transacetylase

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‡ The term de novo as used here refers to synthesis from acetyl-CoA by the pathway involving the intermediate formation of malonyl-CoA.

to acetylcarnitine (11) which is transported to cell sap and reconverted there to acetyl-CoA;* (d) Acetyl-CoA is converted by the condensing enzyme in the mitochondria to citrate. In this last scheme, the acetyl unit is transported to the extramitochondrial compartment either as citrate (13,14) or α-ketoglutarate or glutamate (15) or as all three or any two; these, as the case may be, are then converted to acetyl-CoA by enzymes in the extramitochondrial part of the cell so that the acetyl unit attached to oxalacetate by the mitochondrial condensing enzyme is the same unit as that released by the cytoplasmic citrate-cleavage enzyme (14-16).

In none of these four schemes would carbon originating from the methyl position of pyruvate be removed from acetyl-CoA, and only in scheme (d), where the acetyl-CoA formed from pyruvate condenses with oxalacetate yielding citrate in the mitochondria, would hydrogen from the methyl group of acetyl-CoA be removed. Since the two terminal carbons at the methyl end of a fatty acid chain synthesized *de novo* are derived from acetyl-CoA without loss of methyl-bound hydrogen (17), a comparison of the incorporation of the methyl carbon of pyruvate with that of the methyl hydrogen of pyruvate into the terminal methyl end of the fatty acid chain should tell us whether citrate is an intermediate in fatty acid synthesis. The experiments described here were designed to test this point.

We studied the distribution of ^{3}H and ^{14}C in the fatty acids synthesized by slices of lactating rat mammary glands

* A recent report (12) demonstrating the exclusive localization of carnitine transacetylase in the mitochondria of various animal tissues does not support the view that acetylcarnitine acts as the carrier of acetyl units across the mitochondrial membrane.

TABLE I

CONVERSION OF THE ^3H AND ^{14}C OF (1-^3H,1-^{14}C)-GLUCOSE, (6-^3H,6-^{14}C)-GLUCOSE, L-(3-^3H,3-^{14}C)-LACTATE AND (2-^3H,2-^{14}C)-ACETATE TO FATTY ACIDS BY LACTATING RAT MAMMARY GLAND SLICES

250 mg of lactating rat mammary gland slices were incubated for 3 hours at 37° in 2.5 ml of Krebs-Henseleit bicarbonate buffer (pH 7.3) containing 25 µmoles of labeled and/or unlabeled substrate as indicated below. The gas phase was 95% O_2 and 5% CO_2. The fatty acids (18) and lactic acid (19) were isolated and assayed for ^3H and ^{14}C activity in a Packard liquid scintillation spectrometer by the discriminator-ratio method of Okita et al. (26). Fatty acids were converted to sodium salts and subjected to Kuhn-Roth oxidation. The acetate representing the omega and the omega-minus-one carbon atoms of the fatty acids was isolated and assayed for ^3H and ^{14}C activity. Each value is the average and its standard error of results from 4 separate experiments with different rats.

Substrate		Per cent of utilized isotope recovered as:						
		Fatty acids		Lactic acid		Terminal methyl group of fatty acids		
Labeled	Unlabeled	^{14}C	^3H	^{14}C	^3H	^{14}C	^3H	$^3H/^{14}C$
(1-^3H,^{14}C)-glucose	None	24.7±2.4	20.4±1.8	6.3±0.7	6.5±0.7	4.0±0.5	2.1±0.5	0.54±0.04
(6-^3H,^{14}C)-glucose	None	48.7±5.0	12.4±1.3	8.5±1.0	7.4±0.7	9.5±1.6	5.0±0.9	0.54±0.03
L-(3-^3H,^{14}C)-lactate*	Glucose	74.5±4.2	17.5±0.9	0	0	12.2±2.6	7.3±1.3	0.61±0.03
(2-^3H,^{14}C)-acetate	Glucose	67.4±1.8	23.1±0.6			9.1±1.1	8.2±1.1	0.90±0.02

* L-(3-^3H)-lactate was synthesized by fermentation of (6-^3H)-glucose with L. casei (19); L-(3-^{14}C)-lactate was prepared by reduction (27) of purified (3-^{14}C)-pyruvate (28) with crystalline lactic dehydrogenase and DPNH. The isotopically labeled L-lactates were isolated by paper chromatography in radiochemically pure form.

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from (1-^{3}H,1-^{14}C)-glucose, (6-^{3}H,6-^{14}C)-glucose, L-(3-^{3}H,3-^{14}C)-lactate and (2-^{3}H,2-^{14}C)-acetate (Table I). This tissue was used because of its exceptionally high capacity for lipogenesis. Lactating rats of the Long-Evans strain, 15-16 days postpartum having suckled at least 6 pups, were killed, and their inguinal mammary glands quickly excised. Washed gland slices were incubated in a Krebs-Henseleit bicarbonate buffer containing the isotopically labeled and unlabeled substrates, as shown in Table I. The manner of incubation (15), the fatty acid (18) and lactic acid (19) isolation procedures, and the isotopic assays (18) have been described. The fatty acids were oxidized by a modification (20) of the Kuhn-Roth procedure (21) that yields acetate from only the terminal methyl end. We found, as did D'Adamo (22), that labilization of the hydrogen from the terminal methyl group of the fatty acid occurs to the extent of 30% during this oxidation. Since the extent of labilization was constant under the conditions we used, all values reported in the last column of Table I were corrected for this exchange.

We realized that no valid assessment of the pathway of pyruvate conversion to fatty acids could be arrived at solely from a study of the values for the ratio of $\frac{^3\text{H in total fatty acids}}{^{14}\text{C in total fatty acids}}$ (Table I), since this ratio is largely influenced by (i) the loss of tritium from the internal and carboxyl carbons during the incorporation of malonyl-CoA into fatty acids and (ii) the considerable incorporation into fatty acids of tritium from TPN^{3}H formed from the one position of glucose via the pentose phosphate pathway (23). Thus, any loss of tritium from the terminal methyl group of the

fatty acids would be masked. The use of the ratio $\frac{^3H \text{ in terminal methyl of fatty acids}}{^{14}C \text{ in terminal methyl of fatty acids}}$ shown in the last column of Table I does not suffer from these limitations.

The values for the ratio $\frac{^3H \text{ in terminal methyl of fatty acids}}{^{14}C \text{ in terminal methyl of fatty acids}}$ (Table I) observed in the experiments with (2-^{3}H,2-^{14}C)-acetate were close to unity, whereas those observed in the experiments with (1-^{3}H,1-^{14}C)-glucose, (6-^{3}H,6-^{14}C)-glucose and L-(3-^{3}H,3-^{14}C)-lactate were between 0.5 and 0.6. This indicates that while little tritium from the methyl group of acetate exchanged with hydrogen when acetate was converted to acetyl-CoA, considerable exchange did occur at this position of the acetyl-CoA derived from glucose or lactate. Tritium from the 1 or 6 position of glucose may be lost at the phosphoenolpyruvate stage before this hexose is converted to pyruvate (24), but the similarities in the values for the ratio $\frac{^3H \text{ in terminal methyl of fatty acids}}{^{14}C \text{ in terminal methyl of fatty acids}}$ in the experiments with (1-^{3}H,1-^{14}C)-glucose, (6-^{3}H,6-^{14}C)-glucose and L-(3-^{3}H,3-^{14}C)-lactate suggest that such hydrogen loss in lactating rat mammary gland is of minor importance. A similar conclusion was reached previously (23). The tritium loss we observed in the terminal methyl group of the fatty acids derived from labeled glucose and labeled lactate might also be due to an exchange of hydrogen bound to the methyl carbon of pyruvate when pyruvate is converted to alanine (25). Reversibility of this transamination reaction would result in loss of tritium on the methyl carbon of pyruvate before conversion of pyruvate to acetyl-CoA. However, the calculated values for the ratio

$\frac{^3H \text{ in lactate}}{^{14}C \text{ in lactate}}$ in the experiments with (1-^{3}H,1-^{14}C)-glucose and with (6-^{3}H,6-^{14}C)-glucose were close to unity (columns 5 and 6, Table I). These latter findings indicate that reversible transamination could not account for a value of less than one for the ratio $\frac{^3H \text{ in terminal methyl of fatty acids}}{^{14}C \text{ in terminal methyl of fatty acids}}$ derived from the labeled glucose and labeled lactate (last column, Table I).

Thus, our observations on the conversion of glucose and lactate to fatty acids by the lactating mammary gland are consistent only with the last of the four possibilities presented, i.e., scheme (d), in which the loss of hydrogen that occurs when carbons 1 or 6 of glucose and carbon 3 of lactate are converted to the terminal methyl group of fatty acids is the result, most likely, of citrate formation.

REFERENCES

1. de Duve, C., Wattiaux, R., and Baudhuin, P., Adv. Enzymol., Vol. 24, ed. by R. F. Nord, Interscience Publ., Inc., New York, p. 291 (1962).
2. Wakil, S. J., Ann. Rev. Biochem. 31, 369 (1962).
3. Masoro, E. J., J. Lipid Res. 3, 149 (1962).
4. Abraham, S., Matthes, K. J., and Chaikoff, I. L., Biochim. Biophys. Acta 70, 357 (1963).
5. Lorch, E., Abraham, S., and Chaikoff, I. L., Biochim. Biophys. Acta 70, 627 (1963).
6. Sanadi, D. R., The Enzymes, Vol. 7, ed. by P. D. Boyer, H. Lardy, and K. Myrback, Academic Press, New York, p. 307 (1963).
7. Srere, P. A., Nature 205, 766 (1955).
8. Kornacker, M. S., and Lowenstein, J. M., Biochem. J. 94, 209 (1965).
9. Lowenstein, J. M., The Control of Lipid Metabolism, Biochemical Society Symposium No. 24, ed. by J. K. Grant, Academic Press, London, p. 57 (1963).

10. Wieland, O., and Weiss, H., Bull. Soc. Chim. Biol. 46, 223 (1964).

11. Fritz, I. B., Schultz, S., and Srere, P. A., J. Biol. Chem. 238, 2509 (1963).

12. Beenakkers, A. M., and Klingenberg, M., Biochim. Biophys. Acta 84, 205 (1964).

13. Srere, P. A., and Bhaduri, A., Biochim. Biophys. Acta 59, 487 (1962).

14. Spencer, A. F., and Lowenstein, J. M., J. Biol. Chem. 237, 3640 (1962).

15. Abraham, S., Madsen, J., and Chaikoff, I. L., J. Biol. Chem. 239, 855 (1964).

16. Bhaduri, A., and Srere, P. A., Biochim. Biophys. Acta 70, 221 (1963).

17. Bressler, R., and Wakil, S. J., J. Biol. Chem. 236, 1643 (1961).

18. Abraham, S., Matthes, K. J., and Chaikoff, I. L., Biochim. Biophys. Acta 49, 268 (1961).

19. Bernstein, I. A., and Wood, H. G., Methods in Enzymology, Vol. IV, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, p. 561 (1957).

20. Ginger, L. G., J. Biol. Chem. 156, 456 (1944).

21. Kuhn, R., and Roth, H., Ber. 66, 1274 (1933).

22. D'Adamo, A. F., Jr., personal communication.

23. Abraham, S., Katz, J., Bartley, J., and Chaikoff, I. L., Biochim. Biophys. Acta 70, 690 (1963).

24. Rose, I. A., J. Biol. Chem. 235, 1170 (1960).

25. Oshima, T., and Tamiya, N., Biochem. J. 78, 116 (1961).

26. Okita, G. T., Kabara, J. J., Le Roy, G. V., and Richardson, F., Nucleonics 15, 11 (1957).

27. Kornberg, A., Methods in Enzymology, Vol. I, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, p. 441 (1955).

28. Von Korff, R. W., Anal. Biochem. 8, 171 (1964).

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Effect of Sodium Acetate on Protease Activity[1]

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Abstract

Sodium acetate was shown to have a potentiating effect on some proteases in hydrolysis of milk proteins at acid pH values. This effect was minimal in neutral media, and there was an inhibition of the protease activity at alkaline pH values under some conditions. This was manifest at relatively low acetate concentrations. The response was observed for whey proteins in both liquid and agar media, as well as for casein solutions, with or without previous hydrogen peroxide treatment. It was least evident on nonfat dry milk substrate.

Introduction

During studies on the effect of proteases on variously treated whey proteins, minimal enzymic activity occurred on 1% agar plates containing 1% whey protein, and none was noted on liquid substrate. Cheesman (2) reported that many proteolytic enzymes show a rennet or rennet-like activity on 1% casein in 1% agar with added calcium chloride and sodium acetate as a buffer. This has been confirmed by Ganguli et al. (5, 6). You (7) reported that calcium ions partially inhibited the tryptic digestion of lactoglobulin, due to calcium complexing of the protein molecule. This could not be the case, because in this study when calcium chloride and sodium acetate were added to the whey medium, these "Cheesman additives" increased protease activity. The protease activity was verified on [illegible] casein media and on nonfat dry milk [illegible] 1% protein with and without calcium chloride and sodium acetate. A similar pattern of increased protease activity was observed in these media with the additives. It appeared that the sodium acetate was the stimulation factor which was further investigated.

Materials and Methods

The enzymes were used at a concentration of 1% in distilled water: Miles HT[1], bromelein, pepsin, trypsin 1:250, NBC proteinase, papain, pancreatin, ficin[2], and Maxatase[3]. The more reactive enzymes were particularly studied. Initially, a 1:10,000 solution of rennin[4] and distilled water was used as a control.

A plate method entailed the use of 1% agar with casein, liquid Cheddar whey or nonfat dry milk as substrate at 1% protein concentration. These media were made with and without Cheesman additives, 0.1 M sodium acetate and 10 mM calcium chloride dihydrate. Generally, 1% Difco agar was used. However, in one series of experiments, 0.6% Ionagar Number 2 was used. Twenty milliliters of medium were poured in flat bottom 100-mm petri plates. When solidified, 10-mm wells were cut in the agar with a cork bore. For tests, 0.1 ml of the desired enzyme solution or control solution was placed in these wells, giving a final concentration of 1 mg of enzyme per well. Plates then were incubated at room temperature (25 C) for 48 hr and the diameter of the zone of proteolysis measured. When desired, N HCl or NaOH was used to adjust pH of the medium.

In a tube method, 10-ml portions of whey with various types of heat-treatment and with various additives were tested, 1 mg of enzyme per tube being used. Tests were performed on media with and without 0.1 M sodium acetate. Tubes were incubated at 37 C for four hours and held at room temperature until read visually at 48 hr for evidence of proteolysis.

In further tests, 10-ml portions of 1% casein (Hammersten) in distilled water, with a final pH of 5.30 were incubated with [illegible] of desired enzyme at 37 C for 24 hr. One group was made up to a concentration of 1% hydrogen peroxide, and then treated with catalase after one hour of incubation at room temperature, and a second group was untreated. Controls without enzyme were included. In

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each group, 0.1 M sodium acetate was added to one set, but not to the second set. After incubation, 10 ml of 11% trichloracetic acid were added to each tube. Tubes were allowed to stand at room temperature for 15 min, and centrifuged at 2,500 rpm for 15 min. One milliliter of the supernatant was diluted with 9 ml of distilled water and absorbance was read at 280 mμ for aromatic amino acid concentration as an indication of proteolytic activity as outlined by Wetlaufer (10). Controls were read with distilled water blanks to determine protein breakdown in controls, and these values were read against both distilled water and the controls as blanks. All determinations were made with a Beckman DU spectrophotometer with the hydrogen lamp.

All tests were performed in duplicate and reported as average values of the two results, which were generally within 5% of each other.

Results and Discussion

Table 1 presents typical results of primary observations with 1% whey agar substrate and three types of Cheesman media, using whey, nonfat dry milk or casein as the substrates. Since ionic calcium is known to be present in all four media, the functional variable must be sodium acetate. The major interest was in the proteolytic activity of enzymes on whey proteins, since Cole (3) and Fish et al. (4) have reported that whey proteins resist proteolysis by strongly proteolytic microorganisms. In the case of the enzyme Maxatase it would appear that nonfat dry milk is capable of buffering the sodium acetate effect of increasing proteolytic activity.

Table 2 presents data from studies on the pH effect on sodium acetate stimulation of protease activity on four of the more effective proteases for whey proteins. Whey and nonfat dry milk were used as substrates. Although variations among the four proteases occurred, acetate stimulation was greatest in lower pH levels when it occurred, was minimal at neutrality and decreased at alkaline pH levels to a state of inhibition. The stimulation observed is greatest in the case of whey substrate, as compared with the nonfat dry milk substrate, indicating a greater buffering capacity of nonfat dry milk to protease activity. Since calcium chloride is present in the whey substrate, the interference in tryptic digestion to lactoglobulin reported by Yoe (11) is not borne out. However, no definitive tests were done using an isolated protein as substrate.

Since Bechtle and Schere (1) had shown the Difco agar Number 2 was superior to regular agars for diffusion of large polypeptide antibiotic molecules in antimicrobial sensitivity testing, this agar was used to determine whether enzyme diffusion was a variable. Studies similar to those reported in Tables 1, 2, and 3 were performed using pairs of media prepared from Difco agar Number 2 and Difco agar. Results of all tests were identical, indicating that diffusion is not a variable. This would indicate that the mode of diffusion of large polypeptide protease in agar is not the same as the

Table 1. Sodium acetate potentiation of whey proteolysis by nine enzymes with nonfat dry milk and casein controls.

Enzymes	Substrates: Whey	Nonfat dry milk	Casein
	(zones in mm)		
Miles HT	12[a] / 25[b]	25	25
Bromelin	0 / 0	13	1[illegible]
Pepsin	0 / 0	0	0
Trypsin 1:250	12 / 25	25	25
NBC Proteinase	0 / 12	13	15
Papain	0 / 0	0	12
Pancreatin	12 / 15	15	15
Ficin	0 / 20	20	20
Maxatase	15 / 30	25 / 25	25 / 35
Rennin 1:10,000	0 / 0	25	25
Distilled water	0 / 0	0	0
Media pH	5.60 / 6.00	6.40 / 6.85	6.20 / 6.60

[a] Substrate at 1% protein concentration in 1% agar.

[b] Substrate at above concentration with 0.1 M sodium acetate and 10 mM Calcium chloride dihydrate.

TABLE 2. Effect of pH on protease activity of four enzymes with and without Cheesman additives.

pH	Enzymes: Miles HT	Trypsin 1:250	Pancreatin	Maxatase
	(zones in mm)			
Whey substrate				
5.0	15[a] 26[b]	0 21[c]	0 16	0 23[c]
6.0	22 25	24 27	16 16	18 27
7.0	25 27	35 33	25 23	27 29
8.0	30 27	38 35	32 26	36 31
9.0	34[c] 27[c]	39[c] 37	32[c] 26[c]	36[c] 35
Nonfat dry milk substrate				
5.0[d]	23 28	20 15	17 20[c]	14 14[c]
6.0	26 26	26 26	20 24[c]	21 26[c,e]
7.0	26 26	30 30	22 22[c]	24 30[c,e]
8.0	24 24[c]	28 30[c]	22 24[c]	25 26[c,e]
9.0[e]	24[c] 21[c]	30[c] 28[c]	22 20[c]	26 27[c,e]

[a] Substrate at 1% protein concentration in 1% agar.
[b] Substrate at above concentration with 0.1 M sodium acetate and 10 mM calcium dihydrate.
[c] Zone hazy.
[d] Casein partly precipitated in medium.
[e] Casein partly dissolved in medium.
[f] Double zones with clear, precipitated and clear zones.

[illegible] Cheesman's [illegible] contention that diffusion [illegible] proteolytic enzymes through agar is by an [illegible] mechanism. Recently, Lawrence and [illegible] reported no difference between [illegible] with Ionagar Number 2 and Davis agar [illegible] micro modification of the Cheesman technique.

The effect of sodium acetate concentration [illegible] studied on five enzymes, with typical results presented in Table 3. Again, there was [illegible] from enzyme to enzyme and between [illegible] substrate used. However, the acetate [illegible] occurs in [illegible] media (pH 2.0) [illegible] relatively low concentrations. The 100 mM [illegible] M concentrations. [illegible] activity was observed in acetate concentrations exceeding this level. In 1941 Mahoney et al. (9) reported that whey proteins precipitated by heat and glacial acetic acid were readily hydrolyzed by [illegible]. This might well be the result of [illegible] potentiation of protease at [illegible] pH values.

Table 4 presents [illegible] determination of [illegible] substrate resulting [illegible] casein [illegible] tation of protein. [illegible] readings indicate [illegible]

TABLE 3. Effect of sodium acetate concentration on stimulation of five proteases on two substrates in 1% agar at pH 5.90.

Sodium acetate concn	Enzymes: Miles HT	Trypsin 1:250	NBC proteinase	Pancreatin	Ficin
(mM)	(zones in mm)				
On 1% casein substrate					
100.0	25	25	20	25	25
50.0	25	25	20	25	25
10.0	25	20	20	20	20
5.0	25	25	20	20	25
1.0	25	15	15	15	15
0.5	25	15	15	15	15
0.1	20	15	13	15	15
On 1% whey protein substrate					
100.0	20	25	12	15	15
50.0	15	20	12	15	15
10.0	15	20	0	13	13
5.0	15	20	12	13	12
1.0	15	20	0	13	12
0.5	15	15	0	13	12
0.1	15	15	0	12	0

minations made without hydrogen peroxide treatment indicated no proteolysis of the control casein solution. Some proteolysis was observed in the control casein solution with 1% hydrogen peroxide treatment. Generally, the potentiating effect of sodium acetate in acid medium is evident, with or without hydrogen peroxide treatment of casein, followed by neutralization with catalase. Increased proteolysis with strongly proteolytic enzymes in the case of hydrogen peroxide treatment of casein bears out the observations of Fish et al. (4).

Other tube tests for enzymic proteolysis of whey proteins which had been negative when run without acetate were repeated with 0.1 M sodium acetate. Many tests that were negative in the absence of acetate became positive. The two most active enzymes were Miles HT enzyme and trypsin 1:250. Results with pancreatin seemed to be proportional to its trypsin content. In tube tests, proteolytic activity of enzymes was correlated directly with the severity of heat-treatment that the whey samples had received. This confirms the observation of Haurowitz et al. (7) that denatured proteins are more readily hydrolyzed by trypsin than the native proteins. It would also indicate a trypsin-like hydrolytic activity of the Miles HT enzyme.

TABLE 4. Hydrogen peroxide effect on sodium acetate potentiation of proteases.

Enzymes	No H_2O_2 A 280 mμ	1% H_2O_2 A 280 mμ[a]	1% H_2O_2 A 280 mμ[b]
Miles HT enzyme	0.211[c]	0.231	0.16[illegible]
	0.340[d]	0.371	0.3[illegible]
Trypsin 1:250	0.210	0.316	0.25[illegible]
	0.335	0.375	0.3[illegible]
Pancreatin	0.076	0.181	0.1[illegible]
	0.257	0.361	0.2[illegible]
Maxatase	0.012	0.130	0.0[illegible]
	0.183	0.341	0.2[illegible]
Ficin	0.156	0.084[e]	0.0[illegible]
	0.274	0.099[e]	0.0[illegible]
1% Casein control	0.000	0.066	0.000
	0.000	0.066	0.000

[a] Read against distilled water blank.
[b] Read against casein control blank.
[c] 1% Casein substrate with no sodium acetate.
[d] 1% Casein substrate with 0.1 M sodium acetate.
[e] Casein solubilized by enzyme, but precipitated by trichloroacetic acid.

Recently, Lawrence and Sanderson (8) reported that sodium acetate in the Cheesman assay medium was inhibitory to some of the microbial enzymes tested. This, as well as observations reported here, would indicate two conditions: first, acetate ions should not be present in enzyme test or assay systems unless their effects have been determined, and secondly, acetate ions may have a stimulatory or inhibitory action on an enzyme system, depending on the pH, the substrate and the enzyme involved.

References

(1) Bechtle, R. M. and G. H. Scherr. 1958. A new agar for in vitro antimicrobial sensitivity testing. Antib. and Chemoth., 8: [illegible]

(2) Cheesman, G. C. 1963. Action of rennet and other proteolytic enzymes on casein and casein-agar gels. J. Dairy Res., 30: 17.

(3) Cole, D. H. 1968. Production of a protein hydrolysate by fermentation of milk using *Bacillus cereus* with a study of the proteolytic enzyme involved. M.S. Thesis, Kansas State University, Manhattan.

(4) Fish, N. L., P. J. Pinkston, T. J. Claydon and L. Mickelsen. 1968. Effect of hydrogen peroxide treatment of milk on its [illegible]

teolysis by *Pseudomonas fluorescens*. J. Dairy Sci., 52: 619.

(5) Ganguli, N. C. and V. R. Bhalerao. 1965. Differential action of animal, vegetable and microbial rennets on caseins as revealed by casein agar plate assay method. J. Dairy Sci., 48: 438.

(6) Ganguli, N. C., O. P. Singhal and V. R. Bhalerao. 1966. A simple method for detecting the presence of animal rennet in vegetable or bacterial rennet. Indian J. Dairy Sci., 19: 53.

(7) Hanrowitz, F., M. Tunca, P. Schwerin and V. Goksu. 1945. The action of trypsin on native and denatured proteins. J. Biol. Chem., 157: 621.

(8) Lawrence, R. C. and W. B. Sanderson. 1969. A micro-method for the quantitative estimation of rennets and other proteolytic enzymes. J. Dairy Res., 36: 21.

(9) Malkames, J. P., Jr., H. L. Walter and O. S. Sager. 1951. Enzymatic hydrolysis of whey protein for use in foods. (Mimeo. Rep. USDA) BDI-Inf-118. 4 pp.

(10) Wetlaufer, D. B. 1962. Ultraviolet spectrum of proteins and amino acids. Adv. Prot. Chemistry, 17: 303.

(11) Yon, J. 1954. Influence du calcium sur la cinetique de proteolyse de la lactoglobuline par la trypsine. (The effect of calcium on the rate of tryptic digestion of lactoglobulin.) C. R. Acad. Sci., Paris. 238: 284.

Effect of Aflatoxin B_1 on the in vitro Incorporation of ^{14}C-Acetate into Human Skin Lipids

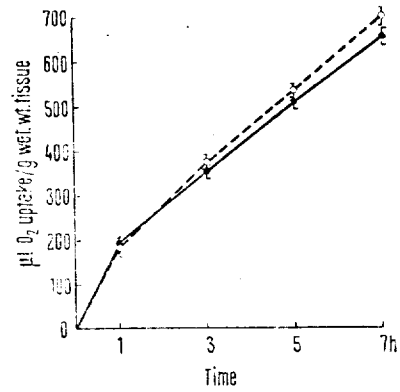
Aflatoxin B_1 is the most potent of the carcinogenic metabolites produced by strains of *Aspergillus flavus*[1]. This fungal product causes liver tumors in numerous species of experimental animals and appears to be a rather specific inhibitor of protein synthesis[2-5]. Protein synthesis is presumably inhibited as the result of the interaction of the toxin and DNA and the resulting inhibition of messenger RNA formation[4].

The manifestations of aflatoxin poisoning appear almost exclusively in the liver, the primary target organ[6]. It has been shown that although aflatoxin is readily absorbed through the skin of the rat, no gross toxic effects occur in this tissue[7]. Contrary to results obtained with rat skin, JOFFE and UNGAR[8] have demonstrated that exposure of rabbit skin to very low levels of the toxin results in severe epidermal lesions. These authors have emphasized the possibility of dermatoses occurring in humans who come in contact with aflatoxins. The possible biochemical lesions in this tissue resulting from aflatoxin poisoning have received little attention. This paper reports the effects of aflatoxin B_1 on the in vitro incorporation of ^{14}C-acetate into human skin lipids.

Materials and methods. Fresh human skin specimens were obtained from the lower abdomen of male Caucasians. The area was anesthetized with 1% lidocaine and the skin removed with a 6 mm dermal punch. Subcutaneous tissue was trimmed and discarded. The skin was rinsed with buffer, blotted dry and weighed, and placed in 15 ml manometric reaction flasks containing 2.0 ml Kreb's-Ringer phosphate buffer, pH 7.4, and 50 µg aflatoxin B_1. The toxin was either dissolved in ethanol or ethanol-propylene glycol (1:1 v/v). Ethanol levels did not exceed 1% of the total volume and the control contained an equal volume of alcohol or alcohol-propylene glycol. The flasks were incubated for 1 h at 37 °C after which 0.5 ml buffer containing 1 µC 1-^{14}C-acetate was added to the incubation medium from the side-arm of the reaction flask. Incubation continued for an additional 6 h at which time the reaction was halted. Respiratory rates were determined manometrically for the total incubation period. The skin specimens were sectioned (50 µ diameter) with a freezing stage microtome and the total lipid extracted by the method of FOLCH et al.[9]. Phospholipids were isolated by the method of SINCLAIR and DOLAN[10] and free sterols, as the digitonides, by the method of SPERRY and WEBB[11]. Neutral fats were isolated after the precipitation of phospholipids and the values in the Table have been corrected for sterols. Radioactivity of each fraction was counted in a liquid scintillation spectrometer. Total content of digitonide-precipitable sterols per unit weight of tissue was determined gravimetrically. Inorganic phosphorous was determined by the method of FISKE and SUBBAROW[12].

Results and discussion. As seen in the Table, aflatoxin effectively inhibits the incorporation of ^{14}C-acetate into the total lipid, phospholipid, free sterol and neutral fat fractions of human skin. Because the rates of isotop incorporation into all the fractions examined were signifi cantly lower than controls, it appeared that the effect o the toxin might be due to general cellular injury. How ever, the respiratory rates of toxin-treated tissues wer not greatly affected and were only 6% less than control at the end of the experiment (Figure).

SHANK and WOGAN[13] demonstrated that marked redu tions in precursor incorporation into liver lipids occurre in ducklings treated with aflatoxin. The lowered rate o precursor incorporation was accompanied with a rise i total lipid content. Similarly, KATO et al.[14] have show



Effect of aflatoxin B_1 on the respiratory rates of human skin. Val represent the mean of 6 experiments ± S.E.M. Respiratory ra are expressed on an accumulative basis.

1 T. ASAO, G. BUCHI, M. M. ABDEL-KADER, S. B. CHANG, E. L. WI and G. N. WOGAN, J. Am. Chem. Soc. 87, 1706 (1965).
2 L. M. ASHLEY, J. E. HALVER and G. N. WOGAN, Fedn Proc. 105 (1964).
3 E. S. BLACK and A. M. ARTSCHUL, Biochem. biophys. Res. Co mun. 19, 464 (1965).
4 J. I. CHEFURKA and K. R. PILES, Biochem. J. 102, 65 (1967).
5 M. C. LANCASTER, F. B. JENKINS and J. McL. PHILP, Nature 1 1075 (1964).
6 G. N. WOGAN, C. S. EDWARDS and R. C. SHANK, Cancer Res. 1729 (1967).
7 R. D. WEI, G. N. [illegible], Experientia 26, 82 (19
8 A. Z. JOFFE and H. UNGAR, J. inv. st. Derm. 42, 404 (1964).
9 J. FOLCH, M. LEES and G. H. SLOANE STANLEY, J. biol. Che 226, 497 (1957).
10 R. G. SINCLAIR and [illegible], J. biol. Chem. 142, 679 (1942).
11 W. M. SPERRY and M. WEBB, J. biol. Chem. 187, 97 (1950).
12 C. H. FISKE and Y. SUBBAROW, J. biol. Chem. 66, 375 (1925).
13 R. C. SHANK and G. N. WOGAN, Toxicol. appl. Pharmac. 9, [illegible] (1966).
14 R. KATO, K. ONODERA and Y. OMORI, Experientia 23, 1026 (19

Effect of aflatoxin B_1 on the incorporation of 1-^{14}C-acetate into human skin lipids

Treatment	Total lipid dpm	10^{-3}	10^{3} µg wet wt	Phospholipid dpm	10^{-3}	µg P	Free sterols dpm	10^{-3}	µg	Neutral fat dpm	10^{-3}	µg wet wt
Control	30,02	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Aflatoxin	25,53	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	5,80	[illegible]	[illegible]	[illegible]	[illegible]	28

Values expressed on the total lipid [illegible] levels respectively. Specific activities [illegible] 6 experiments. Values for free sterols, [illegible]

that aflatoxin B_1 affects the rate of ^{14}C-acetate incorporation into cholesterol in rat liver and in their studies aflatoxin treatment also resulted in elevated levels of cholesterol. These investigators suggested that the inhibitory action of aflatoxin on acetate incorporation into cholesterol was specific and not the result of general hepatic injury.

In our experiments the level of sterol digitonides increased 22% with a concomitant decrease in specific activity of 42%. The increase of lipid and cholesterol content in the previous studies could, as suggested by SHANK and WOGAN, have resulted from failure of transport of these metabolites from the liver. The present in vitro studies, in which transport is not involved, indicate that the toxin affects both the rate of synthesis and turnover of lipids in the skin. Furthermore, the failure of aflatoxin to affect the respiratory rates of skin also suggests that the effects on lipid metabolism are of a more specific nature than that of general toxicity. CLIFFORD and REES[4] have reported comparable results on the respiratory rates of aflatoxin poisoned liver.

Although it is generally concluded that the toxicity of aflatoxin B_1 is tissue specific, the similarities of results from the present study with human skin to those of previous ones of lipid metabolism in animal liver suggest that a) at least this facet of aflatoxin action may be common to all tissues actively engaged in lipid metabolism via similar biosynthetic pathways and b) that this effect of aflatoxin on lipid metabolism is of a specific nature. The relationship of these findings to the possible occurrence of dermatoses in human skin must await further study[15].

Zusammenfassung. Aflatoxin B_1 hindert den in vitro Einbau des ^{14}C-Azetats in die Gesamtlipide, die freien Sterine, die Phospholipide und die Neutralfette der menschlichen Haut. Die Atmungsgeschwindigkeit der toxinbehandelten Gewebe wurde nicht beeinflusst.

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Synthesis *in Vitro* of Steroids by Human Fetal Adrenal Gland Slices*

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Presumptive evidence for the production of steroids by the human fetal adrenal gland has accumulated during recent years. The studies involved mostly analysis of fetal adrenal tissue (1-4) or umbilical cord blood (5-8) for steroid content, the results of which did not unequivocally establish steroid synthesis by the fetal adrenal cortex. Lanman and Silverman (9) demonstrated the conversion of progesterone to corticoids by neonatal adrenal slices. With one exception (1), the several studies were carried out on fetuses during the last trimester of gestation or at term, a period of variable change in the morphology of the fetal adrenal.

The human fetal adrenal cortex is distinguished by the presence of two histologically distinct zones; the definitive (adult) cortex and the fetal zone. The latter comprises about 80 per cent of the total adrenal volume during most of pregnancy (10). Whereas the adult cortex transiently diminishes in size at birth, the fetal zone degenerates rapidly beginning after birth. The separate capacities of these two tissues for steroid production have not been studied.

This report describes the isolation and characterization of several steroids from incubations of human fetal adrenal slices with sodium 1-C^{14}-acetate, and the capacity of both the adult cortex and fetal zone to produce steroids.

METHODS

Quantitative Determination of Steroids—Δ^4-3-Ketosteroid concentrations were determined by measurements of absorbancy at 240 mμ in the Beckman model DU spectrophotometer. Steroids with α-ketolic side chains, when present in amounts less than 0.2 mg. in eluates from paper chromatograms, were measured by the "blue tetrazolium" reaction (11). Dehydroepiandrosterone was determined by the Zimmerman color reaction (12), and Δ^5-pregnene-3β-ol-20-one (pregnenolone) either by the Pettenkofer reaction (13) or gravimetrically.

Radioactivity Determinations—Aliquots of material were transferred to aluminum-cupped planchets. The solvent was allowed to evaporate spontaneously or with the aid of heat from an infrared lamp and a mild stream of nitrogen gas. All radioactivity measurements were made with the Tracerlab model SC-16 or a Robinson windowless gas flow counter for a period of time to assure precision of ±5 per cent or better, the data being corrected to infinite thinness.

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EXPERIMENTAL AND RESULTS

Incubation No. 1

The adrenals from a male fetus, 22 weeks gestational age (21 cm. crown-rump length), were obtained after spontaneous abortion. The tissue was separated into definitive cortex and fetal zone with fine scissors after slicing the glands in 2-mm. intervals. Care was taken to obtain pure fetal zone tissue necessitating contamination of the definitive cortex with some fetal zone tissue. Definitive cortical slices, 1.27 gm., were incubated in fetal serum: 0.9 per cent sodium chloride (1:1) (250 mg. of tissue per 4 ml. of incubation medium) with 0.25 mc. of sodium 1-C^{14}-acetate, and 10 I.U. of ACTH[1] (Corticotropin, Upjohn Company). In an identical manner, 0.54 gm. of definitive cortex and 0.28 and 0.60 gm. of fetal zone were incubated with 500 I.U. of human chorionic gonadotropin (Follutein, Squibb and Sons), 10 I.U. of ACTH, and 500 I.U. of gonadotropin, respectively. The incubations were carried out in a Dubnoff Shaking Incubator (Precision Scientific Company) at 37° ± 1° for 4 hours in air. The definitive cortical slices contained 25 per cent or less of fetal zone tissue.

After incubation and homogenization of the incubates, 0.2 mg. of each of the following steroids was added as carrier to each homogenate: Δ^4-androstene-3,17-dione, dehydroepiandrosterone, 11β-hydroxy-Δ^4-androstene-3,17-dione, corticosterone, cortisol, and 0.1 mg. of pregnenolone. The homogenates were extracted with ethyl acetate and diethyl ether:chloroform (1:1), and purified by heptane-90 per cent methanol partition and paper chromatography as described previously (11). Acetone extraction, silica gel column chromatography, and Girard T hydrazone formation, used in the previous work, were omitted. Of the added carrier steroids 50 to 60 per cent were recovered.

The C_{19} steroids and pregnenolone containing zones from the paper chromatograms were eluted and rechromatographed sequentially twice in the solvent system heptane-propylene glycol (15) and once in hexane-96 per cent methanol (16). Similarly, after elution, corticosterone and cortisol were rechromatographed twice in toluene-propylene glycol (17) and once in toluene-ethyl acetate (9:1)-50 per cent aqueous methanol (16). Before the second chromatography, androsterone and 11β-hydroxyprogesterone were added to the fractions containing dehydroepiandrosterone and 11β-hydroxy-Δ^4-androstene-3,17-dione, respectively. After chromatographic separation and elution, the added steroids

[1] The abbreviation used is: ACTH, adrenocorticotropic hormone.

TABLE I

Radioactivity in steroid fractions obtained from incubations of fetal zone and definitive cortex of adrenals from human fetus, 22 weeks gestational age, after paper chromatographic separation

For details of incubation and chromatography, see text. Activity corrected and calculated back to original amounts of carrier steroid added.

Steroid	Total activity			
	ACTH incubation		Gonadotropin incubation	
	Adult cortex	Fetal zone	Adult cortex	Fetal zone
	c.p.m./100 mg. tissue/hr.			
Dehydroepiandrosterone	92	196	86	135
Δ^4-Androstene-3,17-dione	83	156	62	97
3β-Hydroxy-Δ^5-pregnene-20-one	92	130	147	133

were devoid of radioactivity. Δ^4-Androstene-3,17-dione and pregnenolone, which were eluted from previous chromatograms as one fraction, were acetylated and separated by a third chromatogram.[2]

The total radioactivity in the eluates containing dehydroepiandrosterone and Δ^4-androstene-3,17-dione plus pregnenolone remained constant, *i.e.* within ±10 per cent of the mean value, after two consecutive chromatograms in the two-solvent systems. The sum of the activities of Δ^4-androstene-3,17-dione and pregnenolone acetate accounted for 88 to 95 per cent of the total present in the eluates before separation by acetylation. 11β-Hydroxy-Δ^4-androstene-3,17-dione and corticosterone lost their label slowly. The cortisol fractions unfortunately were lost after the first chromatography.

The analytical data, corrected for carrier steroid recovery, are shown in Table I. The steroid fractions from both the fetal zone and the definitive cortex incubation extracts contained radioactivity. The total radioactivity in the pregnenolone, Δ^4-androstene-3,17-dione, and dehydroepiandrosterone fractions obtained from the fetal zone tissue incubates did not differ significantly from those obtained from the adult cortex incubations. Under ACTH stimulation, fetal zone tissue appeared to incorporate more C^{14}-acetate into these three steroid fractions than definitive cortical tissue; C^{14}-acetate incorporation by the two tissues under gonadotropin stimulation was of the same order of magnitude.

Incubation No. 2

Whole adrenal slices of 38 and 74 mg., from a male fetus, 12 weeks gestational age (8 cm. crown-rump length; fetus removed during therapeutic abortion for carcinoma of the cervix), were incubated with 0.75 I.U. of ACTH and 75 I.U. of gonadotropin, respectively. Each incubation mixture contained 0.22 mc. of sodium 1-C^{14}-acetate; incubation was carried out in 95 per cent O_2:5 per cent CO_2 for 2¼ hours. Other details were the same as for Incubation No. 1.

After incubation and homogenization, the following carrier steroids were added: 2.0 mg. each of Δ^4-androstene-3,17-dione and pregnenolone, 3.0 to 3.3 mg. each of progesterone, 17α-hydroxyprogesterone, Δ^4-androstene-3,11,17-trione (adrenosterone), testosterone, and 11β-hydroxy-Δ^4-androstene-3,17-dione, 4.1 mg. of corticosterone, 6.0 mg. each of dehydroepiandrosterone and cortisol, 1.4 mg. of estrone, and 20 mg. of cholesterol. Subsequent to extraction and purification inclusive of paper chromatography as described previously, 45 to 65 per cent of the added carrier steroids were recovered.

The steroid containing eluates from the paper chromatograms were separated as follows. Testosterone, adrenosterone, 17α-hydroxyprogesterone, and 11β-hydroxy-Δ^4-androstene-3,17-dione were separated by rechromatography in the solvent system heptane-propylene glycol for 96 hours. Δ^4-Androstene-3,17-dione and pregnenolone were separated by digitonin precipitation (18). Estrone was removed from corticosterone by three extractions from chloroform with 0.1 volume of cold 0.1 N NaOH, followed by acidification and ether extraction. The recovered estrone showed no absorbance at 240 mμ.

The isolated steroid fractions from Incubation No. 2 were carried through several crystallizations. After the second crystallization, testosterone and adrenosterone had lost their radioactive labeling. The specific activities (c.p.m. per mg.) of several steroids remained constant during crystallization; however, these values were generally too low (78 to 358 c.p.m. per mg.) to permit further radiochemical purity tests for identification of the isolated steroids. Therefore, C^{14}-steroidal fractions from a third incubation were combined with the individual steroids from Incubation Nos. 1 and 2.

In Incubation No. 3, a total of 0.74 gm. of whole adrenal tissue slices from a female fetus, 18 weeks gestational age (13.5 cm. crown-rump length; fetus obtained during spontaneous abortion), had been incubated with a total of 0.60 mc. of sodium 1-C^{14}-acetate under the same conditions as for Incubation Nos. 1 and 2. Carrier steroids were not added to the homogenate of Incubation No. 3. Extraction and purification inclusive of paper chromatography was similar to that outlined above.

Determination of Radiochemical Purity

The individual pooled steroids were carried through one paper chromatography followed by a final addition of 7.0 to 10.5 mg. of each carrier steroid (3.5 mg. of estrone). Radiochemical purity determinations were carried out by crystallization, countercurrent distribution, derivative formation and paper chromatography. Countercurrent distributions were carried out in a 100-tube glass apparatus (H. O. Post Scientific Instrument Company) according to the procedure of Engel *et al.* (19) and Baggett and Engel (20).

Estrone

The crystalline estrone obtained after one crystallization from diethyl ether-ethyl acetate had a specific activity of 6 c.p.m. per mg., the supernatant, 8 c.p.m. per mg. No further work was carried out on the estrone fraction.

C_{19} Steroids

The specific activities of the three C_{19} steroids (dehydroepiandrosterone, Δ^4-androstene-3,17-dione, 11β-hydroxy-Δ^4-androstene-

Acetic anhydride, 0.05 ml. and 0.06 ml. of pyridine were added to each dried eluate, and allowed to react at room temperature for 20 hours. The solvents were removed by evaporation under nitrogen. The residue was chromatographed in the solvent system heptane-propylene glycol, followed by the elution of Δ^4-androstene-3,17-dione from the chromatogram and recovery of Δ^5-pregnenolone acetate from the overflow.

3,17-dione) observed during repeated crystallizations are [illegible] in Table II. Both the crystalline material and the supernatant gave reproducible (constant) specific activities upon at least two consecutive crystallizations. The isolated steroids were [illegible] subjected to countercurrent distribution. Distribution [illegible] of radioactivity and weight, and of specific activities (Fig. [illegible]) indicated radiochemical homogeneity. The average specific activities, with standard error, were: dehydroepiandrosterone, [illegible] ± 10.8 c.p.m. per mg.; Δ^4-androstene-3,17-dione, 107 ± 4.6; 11β-hydroxy-Δ^4-androstene-3,17-dione, 80 ± 6.8. The precision of the observed counts of 11β-hydroxy-Δ^4-androstene-3,17-dione was 10 to 15 per cent because of low total radioactivity [illegible] background radioactivity ratios. The values obtained after crystallization and countercurrent distribution agreed well with [illegible] other.

C_{19} *Steroid Derivatives*

Dehydroepiandrosterone Acetate—To 3.7 mg. of dehydroepiandrosterone (average specific activity, 349 c.p.m. per mg.), representing the pooled contents of Tubes 34 to 64 after countercurrent distribution, 0.1 ml. of acetic anhydride and 0.2 ml. of anhydrous pyridine were added. After standing at room temperature for 20 hours, the dried crystalline residue was chromatographed in the solvent system heptane-propylene glycol. Dehydroepiandrosterone acetate (1.74 mg.) was eluted from paper, and its identity confirmed by infrared absorption analysis. The specific activity, expressed as free dehydroepiandrosterone, was 130 c.p.m. per mg.

Hydroxylation of Δ^4-Androstene-3,17-dione—Δ^4-Androstene-3,17-dione, 0.5 mg., (average specific activity, 107 c.p.m. per mg.; Tubes 12 to 24) was incubated with 520 mg. of rat adrenal slices by the method described for the incubation of steroids with mouse adrenal tumor slices (21). The oxidation product, 0.155 mg. of 11β-hydroxy-Δ^4-androstene-3,17-dione, after separation and identification by paper chromatography, was found to have a specific activity of 125 c.p.m. per mg.

Oxidation of 11β-Hydroxy-Δ^4-androstene-3,17-dione—An aliquot of the combined contents of Tubes 34–46 from the countercurrent distribution of 11β-hydroxy-Δ^4-androstene-3,17-dione (average specific activity, 80 c.p.m. per mg.) was allowed to react with 3.0 mg. of CrO_3 dissolved in 1.5 ml. of glacial acetic acid for 30 minutes at room temperature with agitation. After reduction of the excess chromic acid with Na_2SO_3, extraction, and chromatographic separation and identification (mobility in heptane-propylene glycol compared to authentic adrenosterone), the specific activity of the oxidation product, 0.30 mg. of adrenosterone, was found to be 72 c.p.m. per mg.

C_{21} *Steroids*

The observed specific activities of the five C_{21} steroids during successive crystallizations are listed in Table III. The crystalline material and the supernatant of pregnenolone, 17α-hydroxyprogesterone, and cortisol exhibited constant specific activities upon at least two consecutive crystallizations. Progesterone and corticosterone lost activity throughout crystallization, the former losing essentially all its labeling. These two steroids were not studied further. Each of the other C_{21} steroids was converted to one or two derivatives, followed by or preceded by countercurrent distribution.

17α-Hydroxyprogesterone—Crystalline 17α-hydroxyprogesterone, 5.5 mg., (average specific activity, 52 c.p.m. per mg.)

TABLE II

Specific activities of Δ^4-androstene-3,17-dione, dehydroepiandrosterone and 11β-hydroxy-Δ^4-androstene-3,17-dione isolated from pooled extracts of human fetal adrenal incubations by dilution carrier technique after repeated crystallization

For details of incubation, see text. Specific activity = c.p.m. per mg.

Crystallization	Δ^4-Androstene-3,17-dione		Dehydroepiandrosterone		11β-Hydroxy-Δ^4-androstene-3,17-dione	
	Specific activity	Solvent*	Specific activity	Solvent*	Specific activity	Solvent*
First						
crystalline	11†	E-H	383	EA-M	1	E-EA
supernatant	126		1		1	
Second						
crystalline	130	E-M	335	E-M	88	E-Et
supernatant	89		368		66	
Third						
crystalline	135	E-H	345	E-H	77	E-Et
supernatant	120		325		72	

* Solvent mixtures used for crystallization: E, diethyl ether; H, hexane; M, 95 per cent methanol; EA, ethyl acetate; Et, 95 per cent ethanol.

† I, impure, contamination with colored material. Specific activities not determined.

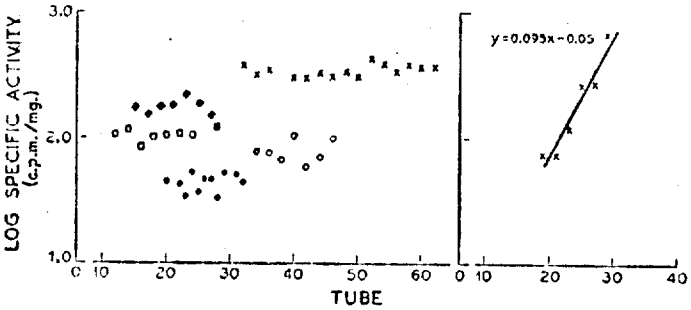

FIG. 1. Solvent system, number of tubes, observed distribution coefficient (K), and percentage recovery for the countercurrent distribution of each steroid were: dehydroepiandrosterone (×): 70 per cent methanol/CCl_4, 100 tubes, K = 0.83, 76 per cent; Δ^4-androstene-3,17-dione (○): C_6H_{12}:ethyl acetate (3:2)/60 per cent ethanol, 50 tubes, K = 0.70, 87 per cent; 11β-hydroxy-Δ^4-androstene-3,17-dione (○): 70 per cent methanol/$CHCl_3$:CCl_4 (1:4), 100 tubes, K = 0.75, 83 per cent; 3β-hydroxy-Δ^5-pregnene-20-one (◆): C_6H_{12}:ethyl acetate (3:2)/60 per cent ethanol, 53 tubes, K = 0.71, 87 per cent; 11β-hydroxy-Δ^4-androstene-3,17-dione (derived from cortisol) (●): 70 per cent methanol/$CHCl_3$:CCl_4 (1:4), 60 tubes, K = 0.76, 65 per cent; Δ^4-androstene-3,17-dione (derived from 17α-hydroxyprogesterone), regression of line (—): × = y = 0.095x − 0.05; C_6H_{12}:ethyl acetate (3:2)/60 per cent ethanol, 53 tubes, 102 per cent. K of Δ^4-androstene-3,17-dione = 0.66, K of radioactive compounds = 1.01, 1.65, 2.05.

was allowed to react with 11 mg. of CrO_3 (as 0.2 per cent solution in glacial acetic acid) at room temperature for 40 minutes with shaking. Water was added, the unreacted chromic acid was reduced with Na_2SO_3, and the oxidation product extracted 4 times with 2 volumes of ethyl acetate. The extract was subjected to silica-gel column chromatography, with the solvent sequence benzene, benzene:ethyl acetate (97:3), (9:1), and (1:1). Δ^4-Androstene-3,17-dione, 1.33 mg., m.p. 172–173° (uncor-

TABLE III

Specific activities of 5 C_{21} steroids isolated from pooled extracts of human fetal adrenal incubations by dilution carrier technique during repeated crystallization

For details of incubation, see text; for abbreviations used, refer to footnotes to Table II.

Steroid	Crystallization: First		Second		Third	
	Specific activity	Solvent	Specific activity	Solvent	Specific activity	Solvent
3β-Hydroxy-Δ⁵-pregnen-20-one						
crystalline	155	E-EA	155	E-M	132	E-H
supernatant	270		88		143	
Progesterone						
crystalline	11	E-EA	55	E-H		
supernatant	63		80			
17α-Hydroxyprogesterone						
crystalline	48	E-EA	37	EA-M	51	EA-H
supernatant	1		52		70	
Corticosterone						
crystalline	78	E-M	33	EA-M	25	EA-H
supernatant	259		104		126	
Cortisol						
crystalline	34	EA-M	48	E-H	32	E-EA
supernatant	41				48	

rected), with mobility identical to authentic Δ⁴-androstene-3,17-dione in heptane-propylene glycol, was obtained from the benzene:ethyl acetate (97:3) fraction. The isolated product possessed a specific activity of 123 c.p.m. per mg. The conversion product was subjected to a 53 transfer countercurrent distribution in cyclohexane:ethyl acetate (3:2):60 per cent aqueous ethanol. The distribution coefficient of steroid absorbing at 240 mμ was 0.60, of three radioactive substances, 1.01, 1.65, and 2.5. The equation describing the relation between the log of the specific activity of Δ⁴-androstene-3,17-dione and the tube number was $y = 0.095x - 0.05$ (see Fig. 1). These data indicate the presence of a substance, in some physical properties similar to, but not identical with, 17α-hydroxyprogesterone.

Pregnenolone—Pregnenolone, 3.1 mg., average specific activity, 131 c.p.m. per mg., was submitted to countercurrent distribution. The results indicated radiochemical homogeneity with an average specific activity of 177 ± 10.6 c.p.m. per mg. (see Fig. 1). The contents of the countercurrent distribution Tubes 15 to 27 were pooled and pregnenolone acetate formed by the procedure described for the acetylation of dehydroepiandrosterone. Crystalline pregnenolone acetate, 2.5 mg., m.p. 148–148.5° (uncorrected), with a specific activity of 144 c.p.m. per mg. (expressed as free pregnenolone) was recovered.

Cortisol—To 7.4 mg. of cortisol (average specific activity, 41 c.p.m. per mg.), a solution of 0.27 gm. of sodium bismuthate in 7.4 ml. of 50 per cent acetic acid was added. The mixture was agitated at room temperature for 40 minutes, and was then diluted with water and extracted quantitatively with ethyl acetate. The extract was filtered, and the filtrate purified by silica gel chromatography as described for 17α-hydroxyprogesterone. 11β-Hydroxy-Δ⁴-androstene-3,17-dione, 4.06 mg., was identified by comparative mobility in the solvent system heptane-propylene glycol, with a specific activity of 44 c.p.m. per mg. (41 c.p.m. per mg. expressed as cortisol) was obtained. This oxidation product was subjected to countercurrent distribution. The average specific activity was 45 ± 2.1 c.p.m. per mg. (expressed as 11β-hydroxy-Δ⁴-androstene-3,17-dione (Fig. 1). The pooled contents of Tubes 20 to 34 (1.33 mg.) were oxidized with CrO_3 by the method described above for the oxidation of 11β-hydroxy-Δ⁴-androstene-3,17-dione. Adrenosterone, 0.36 mg., with a specific activity of 40 c.p.m. per mg. (37 c.p.m. per mg. expressed as cortisol) was recovered and identified by paper chromatography.

DISCUSSION

The results of Incubation No. 1 demonstrate the capacity of both the adrenal definitive cortex (glomerular and fasciculate zones) and the fetal zone of the 22-week-old human fetus to synthesize steroids. Three steroids synthesized by both tissues were partially identified as dehydroepiandrosterone, Δ⁴-androstene-3,17-dione, and pregnenolone. The incorporation of acetate into the two C_{19} steroids, although small, is of the same order of magnitude as that found upon incubation of human adult hyperplastic adrenal tissue (14). Other observations from this laboratory indicate that the total acetate incorporation can be increased by lengthening the duration of incubation from 4 to 12 hours, the incorporation per hour remaining essentially the same. ACTH stimulation of steroid synthesis in fetal zone tissue tended to be greater than in adult cortical tissue.

Dehydroepiandrosterone, Δ⁴-androstene-3,17-dione, 11β-hydroxy-Δ⁴-androstene-3,17-dione, pregnenolone, and cortisol were isolated from the pooled incubations. Testosterone, adrenosterone, and progesterone did not retain their radioactive label. The dissociation of radioactivity from added carrier during initial crystallization (Incubation No. 2) suggest that testosterone and adrenosterone were not formed during the incubations. 17α-Hydroxyprogesterone was contaminated with one or more radioactive substances. These contaminants, after oxidation of 17α-hydroxyprogesterone to Δ⁴-androstene-3,17-dione, were largely separated from Δ⁴-androstene-3,17-dione by countercurrent distribution. However, radioactivity remained associated with all tubes containing Δ⁴-androstene-3,17-dione. Hence, the countercurrent distribution data do not eliminate the possibility that 17α-hydroxyprogesterone was present in small amounts in the incubation extracts. The slow loss of label from corticosterone, a major secretory product in human adult adrenal (22–25), indicates the presence of another substance with physical properties very similar to corticosterone. The decreasing specificity of corticosterone is not considered to indicate its absence; rather, the amounts formed were insufficient for proof of radiochemical homogeneity.

It may be noted that progesterone and 17α-hydroxyprogesterone could not be isolated although pregnenolone was a reaction product. Most likely the conversion of pregnenolone to progesterone is the rate-limiting step in steroid synthesis under the experimental conditions employed. Further, it is suggested

that progesterone is rapidly converted, via 17α-hydroxyprogesterone, to Δ^4-androstene-3,17-dione, cortisol and, possibly, other conversion products. The data of both incubations suggest the formation of significant amounts of other, unidentified steroids.

It is clear that the fetal adrenal cortex during the second quarter of gestation possesses already all the enzyme systems required to convert acetate to steroids. Information regarding the quantitative steroid production pattern could be gained by a comparison of the specific activities of the isolated steroids. Such a comparison cannot be made since the extract from each incubation contributed unequally to the total used for determinations of radiochemical purity. However, the sum of the observations cited here and earlier studies (1, 3) suggest that the steroid production pattern of the fetal adrenal cortex, during the second quarter of gestation differs from that of the human adult adrenal. Although the isolation of cortisol indicates the qualitative presence of the several steroid hydroxylating enzymes present also in adult adrenal tissue, the systems most active in the fetal adrenal result in the predominant formation of other steroids, particularly C_{19} steroids.

Very recently, Solomon *et al.* (26) demonstrated the conversion of progesterone to 17α-hydroxyprogesterone and Δ^4-androstene-3,17-dione by human fetal adrenal tissue, including the fetal zone. The failure to isolate 17α-hydroxyprogesterone in our studies, in addition to the factors discussed above, may be attributed to quantitative differences in experimental design, markedly poorer yields from acetate as substrate, the small amounts of adrenal tissue used, and the absence of carrier 17α-hydroxyprogesterone in the incubation medium.

SUMMARY

Adrenal tissue slices from human fetuses, 12 to 22 weeks gestational age, were incubated with sodium 1-C^{14} acetate and adrenocorticotropin or human chorionic gonadotropin.

Both the definitive cortex and the fetal zone of the fetal adrenal synthesized steroids from acetate. Dehydroepiandrosterone, Δ^4-androstene-3,17-dione and 3β-hydroxy-Δ^5-pregnene-20-one were partially identified in incubation extracts from both tissues.

Dehydroepiandrosterone, Δ^4-androstene-3,17-dione, 11β-hydroxy-Δ^4-androstene-3,17-dione, 3β-hydroxy-Δ^5-pregnene-20-one, and cortisol were isolated by carrier dilution methods from the pooled extracts of three fetal adrenal incubations. Testosterone, adrenosterone, progesterone, and 17α-hydroxyprogesterone were not found in detectable quantities, the two C_{21} steroids probably having been rapidly converted to cortisol, Δ^4-androstene-3,17-dione, and other steroids.

It is concluded that the fetal adrenal during the second quarter of gestation contains the enzyme systems necessary to convert acetate to steroids. Further, the suggestion is made that the human fetal adrenal, although capable of synthesizing the same steroids as the human adult adrenal, produces primarily other steroids, particularly C_{19} steroids, during the second quarter of gestation.

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REFERENCES

1. Bloch, E., Benirschke, K., and Rosemberg, E., *Endocrinology*, **58**, 626 (1956).
2. Kloos, K., and Staemmler, H. J., *Arch. pathol. Anat. u. Physiol. Virchow's*, **324**, 285 (1953).
3. Bloch, E., Benirschke, K., and Doreman, R. I., *J. Clin. Endocrinol. and Metabolism*, **15**, 379 (1955).
4. Gardner, L. I., and Tice, A. A., *Helv. Paediat. Acta*, **12**, 147 (1957).
5. Gardner, L. I., and Walton, R. L., *J. Clin. Invest.*, **33**, 1612 (1954).
6. Migeon, C. J., Keller, A. R., and Holmstrom, E. G., *Bull. Johns Hopkins Hosp.*, **97**, 115 (1955).
7. Salhanick, H., Jones, J. E., and Berliner, D., *Federation Proc.*, **15**, 160 (1956).
8. Forbes, T. R., *Endocrinology*, **56**, 699 (1955).
9. Lanman, J. T., and Silverman, L. M., *Endocrinology*, **60**, 433 (1957).
10. Lanman, J. T., *Medicine*, **32**, 389 (1953).
11. Chen, C., Wheeler, J., and Tewell, H. E., *J. Lab. Clin. Med.*, **42**, 749 (1953).
12. Hellerqvist, A. F., and Koch, F. C., *J. Biol. Chem.*, **135**, 377 (1940).
13. Mason, P. L., Jones, M. E., McCall, P. J., and Gallagher, T. F., *J. Biol. Chem.*, **176**, 73 (1948).
14. Bloch, E., Dorfman, R. I., and Pincus, G., *J. Biol. Chem.*, **224**, 737 (1957).
15. Rubin, B. L., Dorfman, R. I., and Pincus, G., *Recent Progr. in Hormone Research*, **9**, 213 (1954).
16. Bush, I. A., *Recent Progr. in Hormone Research*, **9**, 321 (1954).
17. Burton, R. B., Zaffaroni, A., and Keutmann, E. H., *J. Biol. Chem.*, **193**, 769 (1951).
18. Pincus, G., *J. Clin. Endocrinol.*, **5**, 291 (1945).
19. Engel, L. L., Alexander, J., Carter, P., Elliott, J., and Webster, M., *Anal. Chem.*, **26**, 639 (1954).
20. Baggett, B., and Engel, L. L., *J. Biol. Chem.*, **229**, 443 (1957).
21. Cohen, A. I., Bloch, E., and Cetlozzi, E., *Proc. Soc. Exptl. Biol. Med.*, **95**, 304 (1957).
22. Grant, J. K., Forrest, A. P. M., and Symington, T., *Acta Endocrinol.*, **26**, 195 (1957).
23. Bush, I. E., and Sandberg, A. A., *J. Biol. Chem.*, **205**, 783 (1953).
24. Sweat, M., *J. Clin. Endocrinol. and Metabolism*, **15**, 1013 (1955).
25. Peterson, R. E., *J. Clin. Endocrinol. and Metabolism*, **17**, 1150 (1957).
26. Solomon, S., Lanman, J. T., Lind, J., and Lieberman, S., *J. Biol. Chem.*, **233**, 1081 (1958).

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THE METABOLISM OF ACETIC ACID IN ANIMAL TISSUES

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Although acetic acid has long been suspected to be an intermediate in animal metabolism, the significance of its rôle in biochemical processes has only recently been recognized. The lack of interest in acetic acid metabolism during the past is in part attributable to the inadequacy of methods for identification and for quantitative determination of small amounts of acetic acid, and partly due to the fact that in isolated tissues acetic acid appears to possess only limited reactivity.

Until recently the principal approach to problems of intermediary metabolism has been the balance experiment, i.e., the measurement of the changes in concentration of the reaction product induced by varying concentrations of suspected precursor. The only reactions of acetic acid in animal tissues which could be reasonably well established by balance experiments were the acetylation of foreign amines (1, 2) and the formation of acetoacetic acid (3, 4, 5). A much greater variety of reactions involving acetic acid has been revealed with the aid of isotopically labeled substrates. The utility of the tracer technique for the study of intermediary metabolism derives from the fact that with its aid biochemical conversions become demonstrable irrespective of whether or not the total quantity of the reaction product undergoes a change. Thus the use of labeled acetic acid has been of particular service in detecting acetate formation and in demonstrating its participation in processes which proceed independently of exogenous acetate supply, viz., the biological syntheses of steroids, fatty acids, porphyrin, glucose and uric acid.

In general, it will prove more useful to review a broad segment of intermediary metabolism rather than the metabolism of a single compound. Indeed, the great diversity of biochemical processes, catabolic and anabolic, in which acetic acid is involved would require a much more comprehensive discussion of related subjects of intermediary metabolism than can be given here. Also, the present review is concerned mainly with the metabolism of acetic acid in animal tissues, although reference will be made frequently to processes occurring in other cells. This will be necessary because in many cases the discovery of a biochemical process in microorganisms has preceded and forecast an analogous reaction in animal tissues.

There is evidence to suggest that there exists a biologically active form of acetic acid but its identity has not been established. In the present review the terms acetic acid, acetyl and C_2 unit will be employed, sometimes interchangeably, without any intent of specifying a chemically defined entity.

THE ACETYLATION REACTION. The acetylation of foreign amines and amino acids will be discussed in some detail because this reaction has been of great value for the elucidation of various aspects of acetic acid metabolism in animal tissues.

Acetylation is one of the processes occurring with foreign amines prior to their elimination from the animal body. To what extent acetylation takes place in a given case will depend on the chemical nature and the dosage (6) of the foreign substance and on the species of the test animal. In rats, sulfanilamide is in part excreted unchanged, partly converted to N-acetyl sulfanilamide and oxidized to a hydroxyl compound which can subsequently be conjugated with glucuronic acid. No acetylation of aromatic amines such as sulfanilamide occurs in the dog (7, 13). Also, no unique mechanism exists for the disposal of foreign α-amino acids of the type represented by γ-phenyl α-aminobutyric acid or cyclohexyl alanine. Depending on experimental conditions, the feeding of these amino acids will yield varying proportions of N-acetyl amino acid, hydroxy acid, keto acid and oxidation products derived from the latter (8). Reactions of this kind have been classified as detoxications, a pharmacological term which is generally taken to mean that the body tends to modify foreign substances in such a manner as to render them less injurious to the cell either by increasing their solubility in biological fluids, thus preventing deposition of foreign bodies, or by conversion to derivatives which have less affinity for enzyme systems. The observation that in some instances the toxicity and insolubility of the conversion product exceeds that of the original compound, as in the case of acetylsulfanilamide (9), has made it evident that this type of change undergone by the foreign substance in animal tissues could no longer be adequately described as a detoxication (10, 11). Available evidence indicates that there are but few organic compounds which are entirely refractory to attack by animal cells. The fact that substances are metabolized which are not normally constituents of animal tissues does not necessarily imply the existence of a multitude of enzymes which operate only when the foreign substance is offered to the cell. Thus the coupling of glucuronic acid not only with camphor, borneol or phenol, but also with normally occurring steroid alcohols, or the acetylation of natural as well as of foreign amino acids suggests that these conjugation reactions are not induced by the administration of foreign substances but merely reflect events which are part of the normal intermediary metabolism. The acetylation of a foreign amine may then be attributed to an enzyme which has as its normal function the acetylation of a naturally occurring amine. The enzymes concerned in "detoxication" reactions have not been sufficiently characterized to answer the question whether there exist normal substrates for the enzymes which act upon foreign substances. The enzyme which catalyzes the acetylation of sulfanilamide has been purified (2), but the investigation of substrate specificity has not been extended to normally occurring amino acids. p-amino benzoic acid is acetylated by the same system although at a somewhat slower rate than sulfanilamide.

As an example of a purified enzyme which does not differentiate between a normal and foreign substrate, the l-amino acid oxidase of Green et al. (12) may be mentioned. In the presence of the enzyme, phenylaminobutyric acid, a foreign amino acid, is oxidatively deaminated more rapidly than any of the natural amino acids. Evidently, in this case enzymatic activity is determined

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by the α amino acid configuration and not by whether the amino acid is foreign to the cell.

The view that metabolic occurrences with foreign substances in animal tissues mirror normal events formed the basis for the classical investigations of Neubauer, Knoop and Dakin, who employed phenyl-substituted compounds as models for the study of fatty acids and amino acid metabolism. It attests to the validity of the original concepts that the conclusions arrived at by these investigators some forty years ago have not required any fundamental revision.

Acetylation of amines. Following the early finding of Cohn (13) that p-nitrobenzaldehyde was converted by the dog to acetyl p-aminobenzoic acid, acetylation was more closely investigated with the phenyl derivatives of glycine and of α-aminobutyric acid (8, 14). Although these experiments were designed to study synthesis and degradation of amino acids, they yielded equally important evidence with regard to the participation of acetic acid in intermediary metabolism. Knoop (8) showed that the feeding of racemic phenylaminobutyric acid led to the excretion of an optically active acetyl derivative which he found to be identical with the acetyl compound formed in the animal from γ-phenyl α-ketobutyric acid. Knoop erroneously assigned the unnatural configuration to this acetyl amino acid and concluded that the acid of natural configuration had been converted to the acetyl derivative of the unnatural isomer. Similarly the acetyl derivative of what Knoop believed to be l-phenylalanine was excreted unchanged while the acetyl derivative of the amino acid of opposite configuration, judged from the behavior of the racemic acetyl amino aid, appeared to be readily metabolized (15). In view of these findings Knoop was unable to retain his original suggestion that acetylation was a process of normal amino acid metabolism. However, when the configuration of the stereoisomers of phenylalanine was established by du Vigneaud and Meyer (16) it became clear that the configurations assigned by Knoop were incorrect and that, as was subsequently shown by du Vigneaud and Irish (17), the acetylphenylalanine which was metabolically inert belonged to the d-series. The same authors demonstrated that the acetyl compound excreted after feeding of racemic phenylaminobutyric acid was derived from the l-acid and that it was the amino acid of unnatural configuration which underwent optical inversion. These findings re-established the experimental basis for the acetylation hypothesis. Knoop had originally suggested that acetylation was part of a synthetic process leading to acetylamino acids by condensation of the corresponding keto acids with pyruvic acid and ammonia, in analogy to the in vitro reactions described by Erlenmeyer (18) and deJong (19). That acetylation was not necessarily linked to the amination of keto acids was shown by du Vigneaud et al. (20), who found that phenylamino butyric acid labelled with N^{15} retained during its conversion to the N-acetyl derivative nearly all of the N^{15} originally present, indicating that acetylation of the amino or imino acid had occurred. On the other hand, N^{15} was entirely replaced by normal nitrogen when similarly labeled d-phenylaminobutyric was converted to the acetyl l-acid, a result which pointed to the intermediate formation of phenylketobutyric acid. No need exists to postulate two separate

mechanisms for the acetylation of the isomeric amino acids if it is assumed that acetylation is preceded by inversion of the d- to the l-acid. The same authors also studied the acetylation of phenylaminobutyric acid in animals whose body fluids were enriched with D_2O. One atom of deuterium was introduced at the α carbon atom during the acetylation of both isomeric phenylaminobutyric acids. This can be explained readily in the case of the d-acid as resulting from the reductive amination of the intermediate keto acid by hydrogen derived from the labeled body fluids. On the other hand, introduction of deuterium at the α carbon atom of the acetyl l-acid without simultaneous detachment of nitrogen, as shown by the retention of N^{15}, can be visualized only if there exists an equilibrium between amino and imino acid which permits reversible hydrogenation and dehydrogenation. du Vigneaud et al. (20) therefore suggested a mechanism of acetylation in which condensation of the imino acid with pyruvic acid was the initial step, a scheme akin to that of Knoop (8) with the difference that the imino acid takes the place of keto acid plus ammonia. The formulation of the reaction was based on the assumption that pyruvic acid was the most likely source of acetyl. The subsequent demonstration that acetic acid is an acetylating agent for amines (1, 21) eliminated the necessity to associate the reversible hydrogenation-dehydrogenation, as indicated by the uptake of isotopic hydrogen at the α carbon of acetyl amino acids, with the acetylation reaction. It is more likely that amino-imino acid equilibria are established independently of the acetylation reaction as a general property of amino acids. In some instances the acetylation of foreign amino acids of unnatural configuration seems to proceed more rapidly than oxidative deamination and subsequent inversion. S-benzyl d-cysteine (22), p-bromophenyl- and p-bromobenzyl d-cysteine (23) are in part converted to acetyl d-amino acids. These results provide additional evidence that acetylation can be independent of the amination of keto acids.

Precursors of acetyl groups. From the many attempts to identify the nature of the acetyl precursor by balance experiments with intact animals no clear picture has emerged. For instance, there are reports both that the dietary addition of acetate depresses (24, 25) and increases (26) acetylation of sulfanilamide. In balance experiments with intact animals a significant answer is to be expected only if the exogenous supply of precursor is the limiting factor. The probable acetyl precursors such as acetate, pyruvate, or acetoacetate must be available from metabolic reactions in much greater quantities than are required for the acetylation of the relatively small amounts of foreign amine which can be given to animals so that the dietary addition of precursors is unlikely to influence the yield of acetylated product. Direct evidence concerning the sources of acetyl groups for foreign amines has been obtained from feeding experiments with isotopically labeled test substances. Bernhard has administered to humans and rabbits deuterio acetic acid (21) and deuterio ethanol (27), as a source of acetyl for either sulfanilamide, p-amino benzoic acid or cyclohexylalanine (28). In all cases high concentrations of deuterium were found in the excreted acetyl derivatives, demonstrating the conversion of both acetate and ethanol to acetyl groups for aromatic amines and foreign α amino acids. Similar

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results were obtained with the stereoisomers of phenylaminobutyric acid by Bloch and Rittenberg (29, 30), who studied the quantitative aspects of the acetylation reaction. When equal amounts of labeled acetate are fed together with either d- or l-phenylaminobutyric acid, or with sulfanilamide or p-aminobenzoic acid, the excreted acetyl groups contain in all cases about the same concentrations of isotope. This indicates that acetic acid is an equally efficient source of acetyl groups for the two types of foreign amines represented by phenylaminobutyric acid (α amino acid) and sulfanilamide (aromatic amine). From the data obtained it was concluded that acetic acid is the main source of acyl groups in the acetylation of foreign amines. The level of isotope in the excreted acetyl groups amounts to a relatively small fraction only of the labeled acetic acid administered. This dilution of isotope is believed to result from the mixing of the exogenous labeled acetate with acetic acid arising in intermediary metabolism (30).

The ability of acetic acid to acetylate amines is also clearly indicated by experiments with surviving tissues and tissue extracts. Acetylation of sulfanilamide has been investigated in slices of guinea-pig liver by Klein and Harris (1) and in homogenate and extracts of pigeon liver by Lipmann (2). In these investigations the aromatic amines, sulfanilamide and p-aminobenzoic acid, were used as acetyl acceptors because conjugation could be followed conveniently by determination of the disappearance of diazotizable amine. That acetyl sulfanilamide was the product formed was established by isolation. In these systems acetate was the most potent acetyl donor, while acetoacetate and pyruvate showed a lesser effect. The results suggest that the immediate reactant is either acetic acid itself or a C_2 compound which under these conditions arises more readily from acetate than from pyruvate or acetoacetate. Five times as much acetylsulfanilamide was formed under aerobic than under anaerobic conditions (2). The linking of acetylation with an oxidative process is further illustrated by the finding that the acetate effect is abolished by oxidation poisons such as cyanide or iodoacetate (1). Although oxygen does not enter into the formal equation of the acetylation by acetate, aerobiosis is required to supply the energy for the condensation. Lipmann has demonstrated that acetylation of sulfanilamide takes place also anaerobically if "energy rich" phosphate in the form of adenosine triphosphate is provided (2). It is not likely that the function of adenosine triphosphate consists in the formation of acetylphosphate since the addition of this compound does not elevate the level of anaerobic sulfanilamide acetylation. The enzyme system concerned in the acetylation of these aromatic amines is most abundant in pigeon liver and has been found to be extractable (2).

A different enzyme seems to be responsible for the acetylation of α amino acids by acetate. Bloch and Borek (31) found, with the aid of deuterio acetate, acetylation not only of l-phenylaminobutyric acid but also of the natural amino acids, leucine and phenylalanine. The yields of acetylamino acids were much higher with slices from rat liver than from those of guinea pig or pigeon. After incubation with labeled acetate, amino acid and non-isotopic acetylamino acid as a carrier the quantities of acetylamino acid formed could be calculated from

the isotope content of the isolated acetylamino acid. The amounts of acetyl compound obtained with phenylaminobutyric acid were much greater than with the two natural amino acids, presumably because the acetyl derivative of the foreign amino acid is not further metabolized while the acetylated natural amino acids seem to be short-lived intermediates. Thus, under the same experimental conditions the acetylderivative of a natural amino acid, acetylglycine, was found to be deacetylated.

These results indicate that aromatic amines and both natural and foreign α-amino acids effectively utilize acetic acid as a source of acetyl groups. It is evident that whatever the nature of the C_2 compound which is the immediate acetyl precursor, it can readily be formed from acetic acid itself. The effect of adenosine triphosphate on the acetylation reaction observed by Lipmann (2) suggests that the condensation between the carboxyl group of acetic acid and the amine involves the elimination of phosphoric acid rather than of water. Since the effect of adenosine triphosphate cannot be attributed to the intermediary formation of acetylphosphate, the possibility remains to be explored that phosphorylated sulfanilamide or an acetyl derivative of adenosine triphosphate may be formed as intermediates.

The existence of a second mechanism of acetylation and of another source of acetyl groups is indicated by results obtained with labeled alanine (29, 30). Alanine which contains deuterium at the α and β carbon atoms causes the excretion of deuterio acetyl groups when phenylamino butyric acid is simultaneously administered, but only a slight excess of isotope is found in the acetylation product when sulfanilamide or p-aminobenzoic acid are fed as acetyl acceptors. Since alanine, in contrast to acetate, provides acetyl groups for α amino acids ony, its effect cannot be explained on the basis of intermediate formation of acetic acid. The effect of alanine may reasonably be ascribed to the condensation of a C_3 compound, presumably pyruvic acid, with an imino acid and subsequent decarboxylation of the condensation product to an N-acetyl derivative. In this case the failure of alanine to acetylate aromatic amines can be readily understood since imino acids cannot arise from aromatic amines.

The above result which provides experimental evidence for the acetylation schemes suggested by Knoop (8) and du Vigneaud et al. (20) is of interest in several respects. The reaction:

$$CH_3COCOOH + RCH(NH_2)COOH + \tfrac{1}{2}O_2 \rightarrow RCH(NHCOCH_3)COOH + CO_2 + H_2O \qquad [1]$$

can be assumed to proceed with an overall decrease of free energy so that it becomes unnecessary to postulate the intermediate formation of an active C_2 compound as the immediate precursor of N-acetyl groups. This is in contrast to the acetylation of amines by acetic acid which is an endergonic process and therefore requires coupling with an energy yielding reaction. The conversion of pyruvic acid to acetyl amino acid, by effecting the degradation of a three carbon compound to a C_2 unit without intermediate formation of free acetic acid, offers a possible mechanism for the conversion of pyruvate to the oxidation level of

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acetate, a step in the oxidation of carbohydrate which is little understood at present.

That there may exist a connection between acetylation reaction [1] and the formation of acetoacetate from pyruvate has been pointed out by Krebs (32). The fact that ketone body formation from pyruvate in liver is greatly enhanced in the presence of ammonia (33, 34) can be explained on the basis of acetylamino acid formation from keto acid, ammonia and pyruvate, and subsequent condensation of acetyl groups to acetoacetate. Formation of an N-acetyl derivative from pyruvate has so far been demonstrated only with phenylamino-butyric acid but not with natural amino acids. Also, the ability of N-acetyl groups to yield acetoacetate remains to be tested.

The direct participation of acetoacetate as an intermediate in the acetylation of amines by acetic acid has been considered but appears unlikely in view of the following findings (29, 30): deuterioacetyl groups are formed from butyric acid labeled with heavy hydrogen either at the α or the γ carbon atoms, indicating that both two carbon moieties of the intermediate acetoacetic acid are employed for acetylation. Secondly, deuterioacetic acid which also contains C^{13} at the carboxyl position affords N-acetyl groups in which the relative proportion of C^{13} and D remains unchanged. The formation of acetoacetate from such doubly labeled acetate would entail replacement of deuterium by normal hydrogen, but no loss of C^{13}, as the result of the following reactions:

$$2CD_3C^{13}OOH \underset{+H_2O}{\overset{-HDO}{\rightleftharpoons}}$$

$$CD_3C^{13}OCD_2C^{13}OOH \underset{+H}{\overset{-D}{\rightleftharpoons}} CD_3C^{13}ONa{=}CDC^{13}OOH \qquad [2]$$

$$\downarrow$$

$$(CD_3C^{13}O) + (CH_3C^{13}O)$$

Any acetyl formed by way of acetoacetate should therefore have a diminished $D:C^{13}$ ratio. Since the conversion of acetate to N-acetyl is not accompanied by any loss of carbon-bound deuterium, acetoacetate may be ruled out as an intermediate step. The fact that the $D:C^{13}$ ratio is not affected in a reaction in which acetic acid is utilized also speaks against a rapidly occurring interconversion of acetate and acetoacetate in the intact animal. The significance of this finding with regard to fatty acid catabolism will be discussed later.

Metabolism of acetylamino acids. The occurrence of a process by which acetyl groups can be distributed between amino acids is suggested from data obtained with labeled acetylamino acids (35). These experiments were carried out in order to determine whether the acetyl derivatives of natural amino acids can be biologically deacetylated. Any acetic acid liberated from these compounds should become demonstrable by its capacity to acetylate foreign amines. When the deuterio acetylderivatives of glycine, alanine, leucine or glutamic acid were fed together with phenylaminobutyric acid, deuterium was found in the excreted

acetyl phenylaminobutyric acid, but the level of isotope was very much higher than that obtained after feeding of equivalent quantities of labeled acetic acid. This result was interpreted as showing that the foreign amino acid had received acetyl by direct transfer from the administered acetylamino acid and that in the process no intermediate formation of free acetic acid had occurred. The high isotope concentrations in excreted acetylamine were not obtained when p-amino benzoic acid was the foreign amine. In the transfer of acetyl groups an α amino acid is therefore necessary as an acetyl acceptor. Only the acetyl derivatives of natural amino acids can serve as acetyl donors; the labeled acetyl derivatives of d-alanine, d-leucine and sarcosine failed to yield deuterio acetyl groups. These results paralled those obtained with acetyl d-amino acids in growth experiments and substantiate the conclusion that no biological mechanism is available for the deacylation of acetyl d-amino acids. The fact that α-amino acids are necessary both as acetyl acceptors and as acetyl donors may perhaps indicate that the transfer reaction is coupled with dehydrogenation at the α carbon atom of the amino acids and that in order to allow the reaction to occur the steric configuration of the hydrogen at the α carbon must be that of the natural amino acids. Data on the acetyl transfer reaction have so far been obtained only with phenylaminobutyric acid as acetyl acceptor. *In vivo* the acetyl derivatives of natural amino acids do not accumulate sufficiently to permit their isolation. Transacetylation as a process which operates between two natural amino acids therefore remains to be demonstrated.

Organic chemical reactions representing intermolecular transfer of acetyl groups to amino acids have been known from the investigations of Bergmann et al. Bergmann, du Vigneaud and Zervas (36) found that the acetyl groups of diacetyl diketopiperazine are readily shifted to free amino acids. The reaction proceeds in aqueous solution at room temperature:

Acetyl anhydropeptide + amino acid → anhydropeptide + acetylamino acid [3]

Acetyl transfer was also shown to occur with a histidine derivative which contained the acetyl substituent at one of the imidazole nitrogen atoms (37). Evidently an acetyl group is more reactive when linked to a secondary nitrogen and its transfer to a primary amino group *in vitro* will therefore occur spontaneously. This raises the possibility that the acetyl donor concerned in the biological transfer of acetyl groups may be an N-acetylpeptide rather than a free acetylamino acid. On the basis of these model reactions Bergmann and Zervas (37) suggested that acetylamino acids might play a rôle in the biological formation of peptide bonds. This hypothesis assumes added significance in view of the demonstration that the acetyl derivatives of natural amino acids are biologically formed (31) and that a mechanism exists for the shifting of acetyl groups from one amino acid to another (35).

The formation of an acetylamino acid presumably requires a very similar quantity of energy as the formation of a peptide bond, i.e., 2.5 to 3.0 K cal. (38). This energy can readily be supplied by the acetylation mechanism [1] which involves the conversion of pyruvic acid to N-acetyl. On the other hand, it is

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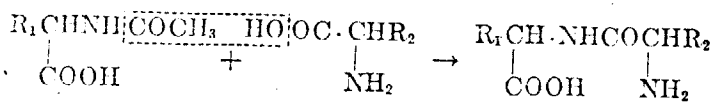
not clear which energy yielding step facilitates the acetylation of amino acids by acetate.

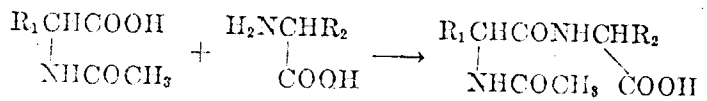
Acetyl amino acids may react with free amino acids in two ways: 1, by acetolysis:



[4]

This reaction should involve only a small change of free energy.

Or 2,



[5]

The second reaction, which would yield an acetylpeptide, is analogous to the acetylation of amino acids by acetic acid, an acetamino acid being the acetylating agent instead of acetic acid. It may be pointed out that as a result of acetylation, the amphoteric amino acids are converted into strong acids (39).

The ability of the animal organism to utilize acetylamino acids is also illustrated by the finding that, with the exception of α-N-acetyl lysine (40), the acetyl derivatives of essential amino acids are nutritionally equivalent to the free amino acids. This has been tested in growth experiments with the acetyl derivatives of tryptophane (41, 42), phenylalanine (43), and histidine (44). The ability of acetylglycine to raise the level of hippuric acid excretion in rabbits above the normal suggests conversion of the acetylderivative to the free amino acid (45). In those instances in which the acetyl amino acid can take the place of an indispensable amino acid, the corresponding α keto or α hydroxy acids will likewise be capable of supporting growth (46). It is therefore not clear whether regeneration of the free amino acid from the acetyl derivative involves direct deacetylation by hydrolysis or whether it is due to a process which regenerates the amino acid by way of the α-keto or α-hydroxy acid. The observation that α-N-acetyllysine cannot support the growth of rats on a lysine deficient diet has been interpreted by Neuberger and Sanger (40) as suggesting rapid oxidation of the ε amino group of lysine prior to deacetylation. However, it is also possible that the α-keto acid is formed directly without passing through free lysine as an intermediate. The α-keto acid of lysine is not known, but it may be presumed to be incapable of substituting for lysine in supporting growth because the α-nitrogen of lysine, in contrast to that of other amino acids, cannot be replaced metabolically by nitrogen from other sources (47) and because d-lysine cannot support growth (48). On the other hand, Neuberger and Sanger (40) found ε-N-acetyllysine to be nutritionally equivalent to lysine, suggesting a process of metabolism in which the acetyl group is removed by hydrolysis to regenerate free lysine. In agreement with this observation Bloch and Rittenberg found deuterio ε-N-acetyllysine to yield acetyl groups for the acetylation of foreign amines with an isotope content to be expected from hydrolysis of the acetyl amino acid (unpublished experiments).

The enzyme systems catalyzing deacetylations show specificity with respect to the spatial configuration of the acetyl amino acids. Thus the acetyl derivatives of d-tryptophane (42, 49), d-phenyl alanine (17) and d-leucine (35) are metabolically inert; acetyl d-phenylalanine has been recovered unchanged from the urine of the experimental animals (17). The sulfur of acetyl d-cystine is resistant to oxidation while acetyl l-cystine is readily metabolized (50). The unavailability of acetyl d-amino acids must be due to the inability of animal tissues to deacetylate because the corresponding free d-amino acids can be utilized and can replace nutritionally their optical antipodes (49, 51, 52). This is further illustrated by the failure of the deuterio acetyl derivatives of d-alanine and d-leucine to yield labeled acetyl for the acetylation of foreign amines (35). Some significance may be attached to the fact that whereas deamination occurs readily with both stereoisomers of most amino acids, only the acetyl amino acids of natural configuration are metabolized by animal tissues.

Acetylation may be associated with the formation of amino acids from keto acids and ammonia but it can also occur subsequent to amino acid synthesis. It is thus conceivable that acetylation may be concerned in the catabolism of amino acids. The following findings are of interest in this connection. The observation already mentioned of Neuberger and Sanger (40) that α-N-acetyllysine cannot replace lysine for support of growth indicates that the compound is not deacetylated but that it may be directly converted to the nutritionally ineffective α keto acid. Secondly, it has been found that on feeding of acetyl-l-phenylalanine to rats, phenylacetic acid can be isolated from the urine in the form of phenaceturic acid. (Unpublished observation of the author.) Since this compound was not obtainable after the feeding of free l-phenylalanine it would appear that the acetyl derivative was deaminated more readily than the free amino acid.

FORMATION OF ACETIC ACID. The first suggestions of a formation of acetic acid in quantity in animal metabolism were contained in experiments of Knoop (53) and Dakin (54) on the biological degradation of phenyl-substituted fatty acids. The excretion of either phenylacetic acid or benzoic acids (or their conjugation products with glycine), depending on the number of carbon atoms in the aliphatic chain of the test substance, was most reasonably explained as resulting from the successive removals of two carbon fragments, by hydrolysis of an intermediate β keto acid. The exact nature of the two carbon fragments could not be ascertained, but chemical considerations pointed to acetic acid or an acetyl compound. Acetic acid has been proposed repeatedly as an intermediate in the main path of carbohydrate oxidation. Thunberg (55) suggested that oxidative decarboxylation of pyruvic acid gave rise to acetic acid, two molecules of which were dehydrogenated to form succinic acid. In the course of their metabolism, several amino acids form intermediates which are identical with products of either carbohydrate or fatty acid breakdown, pointing to protein as a potential source of acetic acid. Thus it was conceivable that the metabolic paths of the three major dietary and tissue components, fat, carbohydrate and protein, would converge at the two carbon stage and that, once this

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stage had been reached, only one common mechanism of oxidation would be required. It is interesting to note that the principal aspects of this theory of intermediary metabolism which, at the time, rested on little experimental evidence, have required revision in a few details only. Experimental evidence in its favor continues to accumulate.

The occurrence of acetic acid in animal tissues. As an intermediate in the main chain of metabolic reactions, acetic acid should continually arise in large quantities. Analysis of tissues and body fluids reveals that in the equilibrium state, acetic acid is present in quantities which are barely detectable (56). Acetic acid in the form of its dinitrophenylhydrazide has been isolated from large quantities of beef liver (57), but it is not clear whether the material thus obtained was an artefact formed during the isolation procedure. Specific methods for the determination of small quantities of acetic acid in biological mixtures are not available; it may also escape detection because it seems to be firmly bound to protein in biological media (58, 59). Nevertheless, even if acetic acid were found to be practically absent, the suggested rôle of acetic acid as a major product of catabolism would not be contradicted. The condition which has to be satisfied, and which applies to all metabolites which lie on the main metabolic paths, is a capacity of the tissue to metabolize the intermediate as rapidly as it is formed. The concentration of the metabolite at any one time will then be irrelevant. The insignificant concentration of acetic acid and other fatty acids of intermediate size in animal tissues has been cited as evidence against the theory of β oxidation (60). If this objection were valid, the same reasoning would apply to numerous commonly accepted intermediates of protein and carbohydrate metabolism, because the demonstration of their presence in normal tissues has not been experimentally feasible.

Evidence for the occurrence of a metabolite which does not normally accumulate in sufficient amounts to permit analytical detection can be obtained with the aid of the isotope dilution method. If the isotopic analogue of a suspected intermediate is administered to an animal, or added to an isolated system, and subsequently recovered either as such or in combined form, a diminished isotope content of the isolated material will indicate that the test substance had merged with identical unlabeled molecules formed in the tissues. Bernhard, in an investigation of the acetylation of foreign amines with labeled test substances (21) found that the acetyl groups of excreted acetyl sulfanilamide contained 5 per cent to 20 per cent as much isotope as the deuterio acetic acid added to the diet. He concluded that the fraction of acetyl groups contributed by exogenous acetate was related to the difference of isotope content in the test substance and excreted acetyl. These findings suggested that the acetyl precursor consisted of a mixture of dietary and endogenous acetic acid. The possibility that the acetic acid excreted by the animal in the form of acetylamine was a representative sample of the acetate existing in the metabolic pool offered an experimental approach to a quantitative determination of this metabolite in the intact animal. From such experiments, Bloch and Rittenberg (30) have concluded that the isotope dilution obtaining in the acetylation process can be attributed to the merging of labeled

dietary acetate with acetate arising in metabolism. The magnitude of the dilution factor indicates that the quantities of acetic acid produced are large.

In order to ascertain the correctness of this conclusion it was necessary to consider the following sources of isotope dilution apart from that caused by endogenous acetate: 1, rate of the acetylation reaction; 2, labilization of the C—H bond in the methyl group of deuterio acetic acid; 3, acetylation by precursors other than acetic acid.

1. Since the acetyl derivatives of foreign amines are not stored by the animal but excreted into the urine soon after their formation, the rate of the reaction need not be considered. The isotope concentration of excreted acetyl is the same irrespective of the length of time during which labeled acetate is administered. 2. In one of the acetylation experiments, a preparation of acetic acid which contained deuterium in the methyl group and a carboxyl group labeled by C^{13} was administered simultaneously with phenylaminobutyric acid. The acetyl group of the excreted acylamino acid contained the two isotopic markers in much smaller concentrations, but the ratio of C^{13}:D had remained unchanged. This finding eliminates loss of deuterium as a source of isotope dilution and also attests to the biological stability of the C—H bonds in the methyl group of acetic acid. 3. If the acetyl groups were derived not only from acetic acid but also from other precursors, such acetyl groups would be non-isotopic and hence dilute the deuterio acetyl groups which originate from labeled acetate. Evidence for the contention that acetate may be the only source of acetyl groups for aromatic amines (sulfanilamide, p-aminobenzoic acid) and the major one in the acetylation of α amino acids has been discussed above. It is based on the finding that in identical feeding experiments, acetic acid effects much higher isotope concentrations in excreted acetyl than any other compound tested. According to Bernhard (27), labeled ethanol is a better source of acetyl than acetic acid, but in the experiments of Bloch and Rittenberg (29) ethanol and acetate were equally effective. On the basis of available evidence it seems likely that alcohol is rapidly oxidized to acetic acid and thus becomes a source of acetyl groups. Still, the possibility exists that a C_2 compound which is readily available from both alcohol and acetate is the immediate acetylating agent.

The finding that alanine is a source of acetyl groups for foreign α amino acids but not for foreign aromatic amines has been attributed to a condensation of pyruvic acid with the imino acid derived from phenylaminobutyric acid. While these results establish the existence of a second source of acetyl groups for α amino acids, they do not invalidate the contention that in the acetylation of aromatic amines acetic acid serves as the sole source of acetyl groups. It was therefore reasonably well established that in feeding experiments with labeled acetate the change in isotope concentration occurring in the acetylation reaction provides a direct measure of endogenous acetate production. By applying the equation for isotope dilution (61), the quantity of acetate formed by the rat in a 24-hour period was calculated to be 15 to 20 mM per 100 grams, or approximately one per cent of the body weight. This value could be in error in either direction if the ingested acetic acid failed to mix adequately with the acetate

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of the metabolic pool, in which case either the dietary material or the endogenous acetate might be employed preferentially for acetylation. However, the following considerations lend support to the correctness of the above calculation: the isotope concentration of excreted acetyl was found to be directly proportional to the total quantity of labeled acetate administered per unit of body weight, and hence the calculated value for acetate production in vivo is independent of dosage variation over a wide range. This finding points to a rapid merging of dietary acetate and the acetic acid formed in the tissues.

In vitro studies by Klein and Harris (1) indicate that the acetylation reaction is confined to the liver. The sample of acetate which becomes trapped by forming acetylamine is therefore representative only of the hepatic acetate pool. Of the acetic acid of extrahepatic origin, only the fraction which escapes oxidation at the site of formation and is dispatched to the liver will be measured in the acetylation reaction. The resultant error cannot be assessed at the moment, because it is not known to what extent acetyl-yielding reactions take place in extrahepatic tissues.

Lorber et al. (62) perfused the isolated heart with labeled acetic acid and found that the latter was apparently diluted by normal acetate in amounts corresponding to those calculated for the intact rat (30).

R. Q. measurements of animals in the fasting state (63) and after hepatectomy (64) have been interpreted as indicating fatty acid oxidation in organs other than the liver. Stadie (65) has calculated that in the animal depleted of carbohydrate stores, the energy requirements cannot be met by hepatic production of ketone bodies. The occurrence of extrahepatic fat oxidation is illustrated by experiments of Lehninger, who showed that palmitic and octanoic acids are metabolized by a heart muscle preparation to yield succinate and ketoglutarate (66). An independent estimate of the size of the metabolic acetate pool has been made on the assumption that a molecule of fatty acid of average size (C_{16}) yields on degradation eight molecules of acetate. With the aid of available data on rates of fatty acid regeneration (67) it is possible to calculate how much acetate should arise in a given period of time if fatty acids were the only source. The quantity thus calculated agrees closely with that obtained experimentally by isotope dilution measurements.

Acetic acid formation from fatty acids. Fatty acid oxidation[1] has been investigated in a variety of systems, viz., in the fasting or diabetic animal, in perfused livers, in various liver preparations, and in the intact animal with the aid of labeled test substances. Under all conditions except those mentioned last, acetoacetic acid is the principal product of the oxidation process. Leloir and Muñoz (68) detected acetic acid in the course of octanoate oxidation by liver, but the amount was insignificant compared with the quantities of ketone bodies which accumulated. Deuel and co-workers (69, 70) found a uniform increase of ketone body excretion with increasing chain length of the fatty acid, administered as salt or ethyl ester, to starving rats. In isolated liver, the yields of

[1] For a detailed consideration of this subject the reader is referred to the review by Stadie (60).

ketone bodies per molecule of fatty acid obtained by different investigators differ widely (71 to 74), depending apparently on the previous treatment of the liver tissue. *In vitro*, fatty acids appear to become increasingly resistant to oxidation as the length of the chain increases. The depressing effect of higher fatty acids on the respiration of isolated tissue, noted by Quastel and Wheatley (71), has been attributed to their surface activity. Lehninger has been able to show, however, that oxygen uptake can be restored and that the higher fatty acids can be oxidized by addition of adenosine triphosphate to the liver system (74). Under the experimental conditions of this investigator the carbon atoms of some even and odd numbered fatty acids (C_8, C_7) could be accounted for as acetoacetate in nearly quantitative yields.

Since acetoacetic acid accumulates as the principal product of fatty acid oxidation, it is not surprising that acetoacetate rather than acetate has frequently been emphasized as the primary oxidation product. On the other hand, recent experiments have furnished clear evidence for the correctness of the principle of β oxidation as proposed by Knoop (53). Stepwise degradation of a biologically occurring fatty acid by elimination of two carbon atoms was first demonstrated by Schoenheimer and Rittenberg (75). Palmitic acid isolated from the tissue fat of animals which had received deuterio stearic acid had a sufficiently high isotope content to show that it arose directly from the C_{18} acid by shortening of the chain. Their data indicated that myristic acid had been similarly formed by subsequent elimination of two carbon atoms from palmitic acid. The further degradation could not be studied because tissue fat contains only traces of the acids with less than 14 carbon atoms.

The two carbon fragment which is detached from the fatty acid chain has not been identified as such, although its intermediate formation has been clearly established. Weinhouse, Medes and Floyd (76) have investigated the oxidation of octanoic acid which was labeled at the carboxyl group with C^{13}, in liver slices. The acetoacetic acid which accumulated as the main product, contained labeled carbon in nearly equal concentrations at the carbonyl (β carbon) and carboxyl positions. Acetoacetate with such isotope distribution could not have resulted from the splitting of the C_8 acid into two four carbon compounds (multiple alternate oxidation), nor could it have been formed exclusively from carbon atoms 5 to 8 of the octanoic acid (classical β oxidation). Acetoacetate must have been formed, at least in part, by random condensation of C_2 fragments.

The ability of fatty acids to yield acetyl groups in the intact animal can be tested by exploiting the fact that foreign amines are converted in vivo into metabolically inert acetyl derivatives. Since acetic acid is an effective source of such acetyl groups, the assumption can be made that the appearance of labeled acetyl amine after feeding of labeled precursor is a measure of acetyl formation. The following deuterio-fatty acids were found by Bloch and Rittenberg (29) to be sources of deuterioacetyl groups: butyric acid, n-valeric acid, isovaleric acid and myristic acid; propionic and undecylic acids failed to yield acetyl. The results obtained were found to conform with the assumption that β oxidation and removal of acetyl groups from the β keto acids was the principal event in the

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metabolism of the fatty acids. It is clear that deuterium is not always an adequate label for the study of carbon chain interconversions, and the results obtained on acetyl formation are in most cases qualitative only. All deuterium attached to the β carbon atoms will be lost by oxidation and further loss of deuterium is to be expected due to keto-enol tautomerism involving the methylene hydrogens adjacent to the keto groups. Thus, α, β dideuteriobutyric acid yields acetyl of much lower isotope concentration than butyrate which contains deuterium at the β and γ positions. If acetoacetate is the intermediate in the butyrate-acetyl conversion, loss of deuterium from the labile α position and therefore a lowered isotope content of the acetyl fragment derived from the carboxyl and α carbon atom of butyric acid would result. The behavior of the two preparations of deuterio butyric acid suggests breakdown of the intermediary β keto acid into two identical acetyl fragments.

The various β keto acids which are formed successively from the higher fatty acids will suffer the same loss of deuterium at the α carbon as the α fragment of butyric acid, so that only the deuterium bound to the terminal carbon remains in stable linkage. In the intact animal, the long chain fatty acids, in contrast to those having a small number of carbon atoms, are only partly catabolized immediately to yield acetyl groups. For example, a large portion of administered myristic acid will be deposited in the tissues as such and will also be converted to palmitic and stearic acids by chain elongation. In this case the deuterium content of excreted acetyl will not be a quantitative measure of the amounts of acetyl produced.

Metabolism of odd numbered fatty acids. The odd numbered straight chain fatty acids are not encountered in animal metabolism but they are oxidized in vivo and in vitro as readily as the corresponding even numbered fatty acids. In isolated liver (72) and in the intact animal (77) the fatty acids possessing an odd number of carbon atoms (except propionic acid) are precursors of ketone bodies although the quantities produced are smaller than those afforded by the neighboring fatty acids with an even number of carbon atoms. Propionic acid is presumably oxidized to a three carbon compound which can be converted quantitatively to carbohydrate. Bloch and Rittenberg have concluded that pyruvic acid cannot be an intermediate in this conversion since deuteriopropionic acid, in contrast to labeled alanine, failed to provide acetyl groups in the acetylation of phenylaminobutyric acid (29). Deuterio n-valeric acid formed labeled acetyl to an extent which indicated that one two-carbon fragment arose per mole of valerate. Cohen found that isovaleric acid is a much more potent source of acetoacetate in liver slices than n-valerate (73). In the intact animal labeled isovalerate yielded more deuterioacetyl than equivalent amounts of the n-valerate (78). From the data obtained, it could not be decided whether the breakdown of this branched chain acid proceeds by way of demethylation to butyric acid or by initial oxidation at the tertiary carbon atom to acetone and a C_2 fragment. Undecylic acid labeled with deuterium at the 10 and 11 positions did not yield deuterio acetyl. β oxidation of this eleven carbon acid may be visualized to lead eventually to a three carbon fragment which contains the isotopic hydrogen but which, like propionic acid, would not be degraded directly to a C_2 unit. The behavior of the odd numbered fatty acids can be readily understood if it is assumed that their metabolism proceeds in a fashion analogous to that of the even-numbered fatty acids with the difference that their terminal three carbon atoms are, like propionic acid, converted to carbohydrate. From an odd numbered acid having n carbon atoms, only n−3 carbon atoms will then be available for ketone body formation so that less acetoacetate will be formed per molecule than from the neighboring even numbered fatty acid.

The demonstration of the ketogenic effect of n-valeric acid (72, 77) has had an important bearing on the development of more recent theories of fat oxidation. This finding could not be adequately explained by either classical β oxidation or by the hypothesis of multiple alternate oxidation. MacKay et al. (77) suggested that the five carbon chain of valerate was degraded to a three carbon and a two carbon unit and that two of the latter combined to yield acetoacetate. It became evident therefore that ketone bodies could be formed by recondensation of two carbon fragments split off from the fatty acid chain as well as by direct oxidation.

Formation of acetyl from amino acids. It is generally believed that α amino acids are catabolized by oxidative deamination to α keto acids and subsequent oxidative decarboxylation to acids containing one carbon atom less. In this case amino acids which yield saturated fatty acids as intermediates should be sources of acetyl groups. This possibility has been studied with the amino acids leucine and valine which contained carbon chains labeled by deuterium (78). Deuterioleucine yielded labeled acetyl groups to the same extent as isovaleric acid indicating that this acid was formed as an intermediate. The ketogenic effect of leucine (73, 79) may be explained on the same basis. Valine and isobutyric acid both are nonketogenic and are also ineffective as sources of acetyl groups. No information is available concerning the steps by which isobutyric acid is further metabolized except that three of its four carbon atoms are convertible to glycogen (80). It should be of interest to ascertain whether the ketogenic action of phenylalanine and tyrosine is associated with acetyl formation, particularly whether the ring structure of these amino acids can be degraded to two carbon units.

Mechanisms of fatty acid oxidation. A consideration of all pertinent data obtained in experiments with intact animals and with *in vitro* systems leads to the conclusion that successive β oxidation, followed by removal of C_2 fragments, is the principal event in fatty acid oxidation. For a number of years undue emphasis was placed on experimental observations which appeared to contradict β oxidation as originally proposed by Knoop. It should be pointed out that the data which led to a criticism of the theory of β oxidation were obtained with either isolated tissues or starving or diabetic animals. Since under these conditions acetoacetic acid and not acetic acid accumulates, the ketone bodies came to be regarded as primary oxidation products not only of the terminal four carbon atoms but of the entire fatty acid chain. Moreover, the theory of multiple alternate oxidation was advanced as a mechanism for fat oxidation

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because, *in vivo* and *in vitro*, the fatty acids containing six and more carbon atoms gave a higher yield of acetoacetic acid than butyric acid, while Knoop's theory provided for the formation of only one mole of keto acid regardless of the length of the chain. However, the formation of ketone bodies in excess of one mole per mole of fatty acid is not at variance with β oxidation if it is ascribed to the recondensation of acetic acid to acetoacetic acid. The occurrence of this reaction was early demonstrated by Loeb (3) and Friedemann (81), who observed acetoacetate formation on perfusing liver with acetate. This finding has been confirmed repeatedly and under a variety of conditions, viz., by Jowett and Quastel and by Leloir and Muñoz in liver slices (72, 68), by Monguio and by MacKay et al. in intact animals (4, 5), and with C^{13} labeled acetate in starving animals by Swenseid et al. (82) and by Medes et al. in liver slices (83). It was recognized by MacKay et al. (5) that the synthetic formation of acetoacetate from acetate provided a basis for a theory of fat oxidation which retained Knoop's basic concept of β oxidation and ascribed the formation of "excess" acetoacetate to a subsequent event, namely, a recombination of two molecules of acetate. The data of Weinhouse et al. with carboxyl labeled octanoic acid (76) demonstrate that acetoacetate can be formed by random coupling of C_2 fragments which are split off from the fatty acid chain. There are, however, two possible pathways which conform with the observed isotope distribution:

$$\text{(a)}\ CH_3(CH_2)_6\,C^{13}OOH \rightarrow 4C_2 \rightarrow 2CH_3C^{13}OCH_2C^{13}OOH \qquad [6]$$
$$\text{(b)}\ CH_3(CH_2)_6\,C^{13}OOH \rightarrow C_2^{13} + C_2 + CH_3CH_2CH_2COOH$$

$$CH_3CH_2CH_2COOH \rightarrow CH_3COCH_2COOH \qquad [7]$$
$$C_2^{13} + C_2^{3} \rightarrow CH_3C^{13}OCH_2C^{13}COOH$$

Reaction (a) does not indicate the mechanism which yields the four C_2 fragments from the fatty acid chain; it implies that all of the acetoacetate is of synthetic origin. Reaction (b) is a modified version of classical β oxidation and retains the view that the four terminal carbon atoms are a direct source of acetoacetic acid. In this case the accumulated acetoacetic acid would be a mixture of "primary" and synthetic molecules. In similar experiments with carboxyl labeled butyric acid the resulting acetoacetate contained an unequal distribution of C^{13} at the carboxyl and carbonyl carbon atoms (85). This acetoacetate could therefore not have been formed by random coupling of two equivalent C_2 fragments. Medes et al. (85) have pointed out that their data can be represented by the following equation:

$$CH_3CH_2CH_2COOH \rightarrow CH_3COCH_2COOH \rightleftarrows 2CH_3COOH \qquad [8]$$

In this case rapid interconversion of acetate and acetoacetate has to be postulated. This is true for the synthesis of acetoacetate in liver (84) and kidney (86). The reverse reaction was found by Lehninger (59) to proceed slowly in muscle mince, but it is not clear whether in liver and kidney acetoacetate is split at a sufficient rate to account for the redistribution of isotope in accordance with the above equation.

A similar explanation may apply to the results obtained by Morehouse (87) and by Morehouse and Deuel (88), who fed two preparations of deuterio butyric acid and deuterio caproic acid to starving rats and isolated β-hydroxybutyric acid from the urine. The excreted hydroxybutyrate contained nearly twice as much deuterium when β-γ-dideuteriocaproate was fed as the acid excreted after administration of caproate labeled at the α and β positions. It is evident that a splitting of the hexanoic acid into three two carbon units followed by recondensation to acetoacetic acid should yield hydroxybutyric acid containing the same isotope concentration in both cases. On the other hand, if the excreted β-hydroxybutyric acid was derived mainly from "primary" acetoacetate representing the intact chain of carbon atoms 3 to 6 of caproate, and was formed only in part by recondensation of C_2 units, then β-γ-dideuteriocaproate should yield a hydroxybutyric acid of higher deuterium concentration than α-β-dideuteriocaproate.

Stadie (60) has pointed out that the data obtained by Weinhouse et al. with labeled octanoate are not inconsistent with the theory of multiple alternate oxidation if the random distribution of labeled carbon is the result of a splitting and reformation of acetoacetate. Since it is agreed that the formation of a C_2 fragment is an obligatory step in the breakdown of fatty acids, it remains to be decided whether acetoacetate is the primary oxidation product or whether its formation represents an alternative path for the disposal of C_2 fragments which are initially formed.

Some indications as to the rôle of acetoacetate in fatty acid oxidation are contained in experiments on the acetylation of foreign amines by acetic acid (30). It has been pointed out that the results from these experiments eliminate acetoacetic acid as an intermediate in the conversion of acetate to N-acetyl groups. From the same analytical data it can be concluded[2] that in the intact animal a rapid interconversion of acetate and acetoacetate is unlikely to occur. Doubly labeled acetic acid ($CD_3C^{13}OOH$), when fed and recovered as acetyl phenylaminobutyric acid, retains deuterium and C^{13} in identical relative concentrations. Had acetate been converted to acetoacetate and regenerated, replacement of deuterium by normal hydrogen and loss of deuterium by enolization should have altered the $D:C^{13}$ ratio. The accumulation of acetoacetic acid in fatty acid oxidation in vitro, or in the urine of animals in ketosis may therefore be ascribed to circumstances which interfere with the normal disposal of acetyl or acetate. The isolated liver or liver preparations as ordinarily employed contain little available carbohydrate, and therefore lack the requisite dicarboxylic acids for the oxidation of the intermediates of fatty acid breakdown. Liver tissue appears to have only a limited capacity to oxidize fat beyond the C_2 stage but dispatches the products to extrahepatic tissues for complete combustion. In the isolated liver acetyl will therefore accumulate and become stabilized in the form of acetoacetate. This point is clearly illustrated by the experiments of Lehninger (89) who found that in preparations of washed liver cells, acetoacetate is the

[2] The author is indebted to Prof. D. L. Thomson for having called attention to these implications contained in the experimental data.

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sole product of octanoate oxidation, but that on addition of fumarate the quantities of acetoacetate are diminished in favor of α-ketoglutarate, succinate, and citrate. It becomes unnecessary then to assume that acetoacetate lies on the main path of fat oxidation. Acetoacetic acid is utilized under a variety of conditions, but this is probably preceded by its splitting into two carbon units.

Formation of acetyl from pyruvate. There exists at present no conclusive evidence to indicate whether pyruvic acid enters the citric acid cycle by combining directly with oxaloacetic acid or whether this condensation is preceded by a conversion of pyruvate to an intermediate which has the oxidation level of acetic acid. Formation of acetate from pyruvate can be demonstrated to occur in animal tissues, either aerobically (90, 91, 92) or anaerobically by dismutation (90, 91, 93) but this reaction is not believed to represent an important pathway, because under the same conditions acetate itself is oxidized relatively slowly. It is considered more likely that the oxidation product of pyruvate is represented by a two carbon compound which is metabolically more reactive than acetic acid itself. A phosphoroclastic splitting of pyruvate into acetyl-phosphate and formate (or $CO_2 + H_2$) as it occurs in bacteria (94) has not been observed in animal tissues.

According to Barron et al. (95), acetate can be shown to accumulate when liver or kidney slices oxidize pyruvate in the presence of fluoroacetate. According to these investigators, this compound seems to inhibit the oxidative disappearance of acetic acid by competing with acetate for the enzyme. The accumulation of acetate indicates that fluoroacetate may exert its effect by blocking condensations involving the reactive acetyl compound, which may then become stabilized in the form of acetate.

In the presence of malonate, pyruvate oxidation by liver slices or mince affords acetoacetate in quantities up to 25 per cent of the pyruvate metabolized (33, 34, 96). Pyruvate is quantitatively converted to acetoacetate by the washed liver cell preparation of Lehninger (66). When fumarate was added to the same system, acetoacetate formation was suppressed in favor of an increased yield of di- and tri-carboxylic acids. The in vitro conditions which affected the relative yields of ketone bodies and polycarboxylic acids respectively were the same whether octanoic acid or pyruvate served as substrates. On the basis of these findings it would appear that a common oxidation product is formed from both compounds. A view which has been widely accepted is that an acetyl-like two carbon unit forms the intermediate step at which the pathways of carbohydrate and fat metabolism converge.

However, certain difficulties are encountered when an attempt is made to correlate the ketone body formation from pyruvate *in vitro* with the metabolic fate of fat and carbohydrate in the intact organism. It is well known that in the starving or diabetic animal fatty acids enhance ketone body formation, whereas pyruvate or lactate reduce ketosis. Evidently, under these conditions, the two substances do not enter a common path. It is possible that acetoacetate formation from pyruvate is the favored reaction under special conditions in vitro, but it may still be quantitatively insignificant for the pyruvate metabolism of the intact animal. The hypothesis that a C_2 fragment arises in the oxidation of both carbohydrate and fat would seem to be untenable unless it is assumed that the C_2 fragments are not identical.

The contrasting behavior of labeled fatty acids and pyruvate in the acetylation of foreign amines in intact animals leads to similar conclusions (30). While acetate is an effective source of acetyl for both aromatic amines (sulfanilamide) and α-amino acids (phenylaminobutyric acid), alanine (pyruvic acid) can yield acetyl to foreign α-amino acids only. Moreover, labeled alanine, unlike acetate or butyrate, does not serve as a precursor for cholesterol, either *in vivo* (30) or *in vitro* (97). If pyruvate were converted to the same C_2 fragment which is active in the acetylation of foreign amines and in cholesterol formation, labeled alanine or pyruvate should show the same effect as acetate. Recent experiments by Sonne et al. (98) on uric acid formation contain similar evidence. The feeding of $CH_3C^{13}OOH$ resulted in the incorporation of heavy carbon at carbon atoms 2 and 8 of the purine nucleus. No significant concentrations of C^{13} appeared at these positions when $C^{13}H_3C^{13}HOHCOOH$ was fed, indicating that lactate is not broken down to the two carbon compound employed in uric acid synthesis.

It is conceivable that the differences observed are quantitative rather than qualitative, but it is not likely that the bulk of pyruvate is oxidized by way of the same C_2 fragment which arises in the catabolism of fatty acids. Bloch and Rittenberg have concluded that the amounts of acetate produced in the intact rat can be accounted for fully by the oxidative metabolism of fatty acids and ketogenic amino acids (30). These data also would seem to exclude any considerable breakdown of pyruvic acid by way of acetyl as an intermediate.

Acetylmethylcarbinol (acetoin) has been obtained as a product of the anaerobic metabolism of pyruvate in micro-organisms (99) and in animal tissues (heart muscle) (100). The process is believed to involve oxidative decarboxylation to acetaldehyde, condensation of the latter with a second molecule of pyruvic acid, and subsequent decarboxylation of the condensation product. A scheme involving the following steps: pyruvate → acetoin → diacetyl → acetyl phosphate → acetate—has been proposed by Doisy and Westerfeld (24) on the basis of the effect shown by acetoin in the acetylation of p-aminobenzoic acid. The relatively high toxicity of acetoin for animals (101) and the fact that it is in part excreted after reduction to butyleneglycol (102) seems to preclude the possibility that the above scheme represents a major pathway of pyruvate metabolism.

The formation of acetate from pyruvate by an indirect route involving the splitting of intermediates of the tricarboxylic acid cycle has become a possibility in view of the following observations. The fermentation of citrate by yeast (103) and by various bacteria (104, 105, 106) has been found to yield 1 to 2 moles of acetate and smaller amounts of formate and succinate. These results have been interpreted to suggest the following series of reactions: citrate → acetate + oxaloacetate → pyruvate + CO_2 → acetate + formate. The first of these steps, i.e., the reversal of the condensation of acetate and oxaloacetate is believed by Lipton and Barron (107) to be responsible for the positive effect shown by citrate in the acetylation of choline in brain extracts. The reversibility of the initial

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condensation reaction in the tricarboxylic acid cycle has not been tested in other systems of animal origin.

Slade and Werkmann (108) have studied the conversion of succinate to acetate by *Aerobacter indologenes* with the aid of C^{13}. Although these authors conclude that their data indicate a splitting of succinate into two molecules of acetic acid, it is equally possible that the acetate arose not from succinate directly but by degradation of either α-ketoglutarate or of a tricarboxylic acid formed from succinate. Irrespective of the pathway involved, these results suggest a possible route for the conversion of carbohydrate intermediates to acetate and fatty acids which circumvents the decarboxylation of pyruvate itself.

Utilization of acetic acid for synthetic reactions. The administration of labeled acetic acid to animals has been found to result in the incorporation of isotope into a variety of tissue constituents: glycogen, cholesterol, fatty acids, the dicarboxylic amino acids, protoporphyrin and uric acid. It thus appears that in the biological formation of the cell constituents acetic acid is of general importance as a source of carbon atoms. It has become increasingly evident in recent years that body constituents of high molecular weight are synthesized by condensation of numerous small sized units rather than by the utilization and rearrangement of preformed large molecules.

In a few cases only the animal organism depends exclusively on an exogenous supply for the maintenance of cellular composition (vitamins, essential amino acids and essential fatty acids). The majority of the tissue constituents can be derived not only from unchanged dietary material but can also arise by synthesis from small molecular breakdown products which are formed in intermediary metabolism. The ability of the animal organism to keep its tissue composition constant under a variety of dietary conditions is an expression of the fact that the required elementary building stones are available from a number of sources.

Older suggestions that cholesterol might be formed either by modification of the preformed steroid structure which is offered to the animal in the form of squalene (109) or plant steroids (110), or by folding of long chain fatty acids (111) have given way to the view that a total synthesis from numerous small units is involved (112). In the synthesis of long chain fatty acids from carbohydrate the sugar molecule is presumably not utilized as such but only after its degradation to C_2 or C_3 fragments. In the formation of the tetra pyrrole structure of porphyrin, two C_2 compounds, acetic acid (30) and glycine (113, 114), serve as a source of carbon and nitrogen and not the preformed five-membered ring structures of proline or pyrrolidonecarboxylic acid (from glutamic acid). The investigations on the biological synthesis of these high-molecular tissue constituents have so far been carried out mainly with intact animals. It is evident that for the synthesis of the more complex molecules a high state of cellular organization will be required. For this reason virtually no attempts have been made to investigate the enzymatic processes concerned in the synthesis of the substances mentioned. It has not been possible to identify any of the intermediate steps in the biosynthesis of fatty acids, cholesterol or porphyrin, nor do indications exist that such intermediates occur normally in measurable quantities.

The utilization of acetic acid for biological syntheses has been demonstrated by the feeding of labeled acetic acid to animals and isotope analysis of the body constituents isolated from the tissues. Insight into the reaction mechanisms has in some cases been gained by determining the location and distribution of isotope at various positions of the synthetic product, but the accessible information is often limited by the lack of suitable methods of degradation. Wood et al. (115) have employed chemical and microbiological degradation procedures for glucose which permit separate isotope analysis of carbon atoms 3 and 6 and of fractions which represent carbon atoms 1 and 6, 2 and 5, and 3 and 4 respectively. Methods suitable for determining the deuterium concentrations at separate positions of deuterioglucose have been described by Stetten and Stetten (116). Although procedures are available for successive degradation of the higher fatty acids they are not applicable to small quantities of biological material. However, analysis of the carboxyl group and of the two moieties obtainable by degradation of oleic acid have yielded valuable information on the mechanism of fatty acid synthesis (117). The distribution of isotope in deuteriocholesterol has been determined by degradation of the steroid molecule into nucleus and side chain (118). No experiments of this type have so far been carried out with protoporphyrin formed biologically in the presence of labeled acetate.

Although it may be feasible to study details of reaction mechanisms by employing test substances which contain more than one isotopic marker, there are certain limitations to the experimentation with intact animals. If, in a synthetic process, acetic acid did not take part as such but in the form of a functional derivative (acetylphosphate, acetylamine) and if the substituent were eliminated in the course of the condensation, isotope analysis of the reaction product may not reveal whether acetic acid reacted as such or in a combined form. On the other hand, experiments with intact animals are indispensable in order to establish the occurrence and the quantitative significance of a process which has been observed in an isolated system.

Glycogen. According to classical concepts, a glycogenic compound is a substance which either increases glycogen deposition in the liver of a fasted animal or enhances glycosuria in the diabetic animal. Acetic acid does not satisfy these criteria, since its feeding neither increases glycogen deposition nor glucose excretion (119). On the other hand, isotope experiments have shown that acetic acid can provide carbon atoms for all positions of the glucose molecule (120, 121). No inconsistency need be seen in these findings, if (a) the meaning of the term precursor is more clearly defined and if (b) it is recognized that the terms glycogenic and ketogenic apply only to special experimental conditions. The revision of classical concepts necessitated by the results obtained with tracer substances has been discussed by Buchanan and Hastings (122) and by Wood (123). If one disregards reactions in which a substance effects an increased formation of a product by stimulation rather than by direct conversion, then two types of precursors have to be considered: substances which afford a net increase of the quantity of product, i.e., reactions in which the quantity of precursor is the limiting factor, and secondly substances which are incorporated either totally or

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in part into reaction products but which are without effect on the rate of its formation. Compounds which in classical terminology are classified as either glycogenic or ketogenic belong to the first group while acetic acid with respect to glycogen formation belongs to the second category. Although, as Lorber, Lifson and Wood (121) have shown, acetic acid provides carbon atoms for all positions of the glucose molecule, it probably cannot by itself form hexose (or pyruvate) but only by entering the tricarboxylic acid cycle. Through a series of transformations the tricarboxylic acid yields pyruvate, which is the obligatory intermediate in glucose formation. Acetate utilization for glycogen synthesis is therefore not regulated by the availability of acetic acid but depends on tricarboxylic acid formation and regeneration of pyruvate from the latter. On the other hand the conversion of pyruvate to glycogen does not require the intervention of the citric acid cycle and as a result pyruvate and compounds which are directly convertible to pyruvate will be precursors of glycogen in the sense that they can increase the net amount of glycogen formed. It is evident that the terms glycogenic and ketogenic apply only to conditions in which a change of carbohydrate concentration is observable, i.e., in the starving or diabetic animal. From its behavior in isolated liver, pyruvic acid could be classified as a ketogenic substance, since under such conditions it can be converted in part (124, 33, 90, 96) or quantitatively (125) to acetoacetatic acid. Acetic acid is ketogenic since it increases ketone body excretion in the starving or diabetic animal (5), but as isotope studies have shown, it is also a source of carbon atoms for glucose. The appearance of acetate carbon in all positions of the glucose molecule has been ascribed by Wood (115) to a series of steps by which a tricarboxylic acid formed from acetate and oxaloacetate is degraded to pyruvate by way of α-ketoglutarate and the C_4 dicarboxylic acids. If $C^{13}H_3C^{13}OOH$ is employed the resultant pyruvate will be labeled at all three positions but it cannot contain more than half of the isotope concentration present in the acetate which was utilized. On tracing the labeled carbon through tricarboxylic acid, α-ketoglutarate and the dicarboxylic acids to succinate, it becomes clear that only two of the four carbons in the dicarboxylic acids originate from acetate but the isotopic carbon becomes evenly distributed over the entire carbon chain when the asymmetry of the molecule is lost at the succinate stage. Pyruvate will therefore contain C^{13} at all positions. It can be concluded that although acetate carbon appears in all positions of glucose not more than half of the carbon atoms of a glucose molecule can have originated from acetate, the remainder being supplied by oxaloacetate. Acetate per se is therefore incapable of forming glucose.

Carboxylation of acetic acid to pyruvic acid cannot be an important pathway for the utilization of acetate in glycogen formation because in this case the isotope distribution in glycogen would be expected to differ from that actually observed (121).

Fatty acids. McLean and Hoffert (126) have studied lipid formation in yeast from acetate and have concluded from balance data that both steroids and neutral fat could be synthesized directly from acetate without intermediate formation of carbohydrate. In the presence of sulfite steroid synthesis was

inhibited, but fat continued to be synthesized. This suggested that acetaldehyde might be an intermediate in the synthesis of yeast steroids while acetate could condense directly to fatty acids. In their experiments on the metabolism of deuterioacetate in yeast, Sonderhoff and Thomas (127) found the following concentrations of deuterium in the various fractions: carbohydrate 1.6 per cent; fatty acids 14.7 per cent; unsaponifiable 30 per cent, indicating that carbohydrate was not an intermediate in the conversion of acetate to lipids.

Wood et al. (128) have demonstrated the bacterial utilization of acetic acid for the synthesis of lower fatty acids. In the presence of $CH_3C^{13}OOH$, the butyl alcohol formed in the fermentation of *Cl. butylicum* and of *Cl. acetobutylicum* was found to contain C^{13} at carbon atoms 1 and 3. Since these organisms can reduce butyric acid to butanol, the following series of reactions is suggested: 2 acetate → butyrate → butanol.

The fermentation of alcohol and acetate in *Cl. kluyveri* has been studied by Barker, Kamen and Bornstein (129). The butyric acid formed in the presence of $CH_3C^{14}OOH$ contained approximately equal quantities of heavy carbon in the carboxyl and β positions; in caproic acid equal amounts of C^{14} were presumably present at alternate carbon atoms since the carboxyl group accounted for about one-third of the total isotope content of the hexanoic acid. Important information with respect to the mechanism of fatty acid synthesis in these organisms was obtained by the same authors. Isotopic caproate formed in the presence of $CH_3CH_2CH_2C^{14}OOH$ and normal ethanol contained isotope but this was not present in the carboxyl group. The active C_2 unit derived from ethanol must therefore have condensed with the carboxyl group of butyric acid. Moreover, a breakdown of butyrate to C_2 compounds prior to formation of the C_6 acid cannot have occurred since in this case the carboxyl carbon of caproic acid should have contained the carbon isotope.

Rittenberg and Bloch (117) have fed mice and rats acetic acid which contained C^{13} at the carboxyl position and deuterium in the methyl group. The fatty acids isolated from the tissue lipids contained both isotopes in excess of the concentrations which could have resulted from the oxidation of acetate to $C^{13}O_2$ and D_2O and incorporation of deuterium from the body fluids or C^{13} by a reaction involving CO_2 assimilation. The body fluids of the mice contained 0.09 per cent D and the respiratory CO_2 contained 0.066 per cent C^{13} while the saturated fatty acids isolated from the liver lipids contained 0.42 per cent D and 0.16 per cent C^{13}. As the fatty acids contained both C^{13} and deuterium it is clear that the acetic acid molecule as such had been utilized in the synthetic process. From acetic acid containing isotopic carbon in the carboxyl group, a fatty acid containing C^{13} only at one out of two carbon atoms is to be expected. The incorporation of isotope into the fatty acids could result from the following processes: 1, acetic acid elongates the fatty acid chain by adding at the carboxyl end. In this case the presence of isotopic carbon should be confined to the carboxyl carbon of the fatty acid and decarboxylation should leave a non-isotopic residue; 2, If acetic acid condensed with the ω carbon of the fatty acid decarboxylation should yield CO_2 which contains no excess of isotope; 3, acetate carbon is uniformly

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distributed over the fatty acid chain. C^{13} should then be present at the odd-numbered carbon atoms. The CO_2 obtained on decarboxylation of the fatty acid actually contained about twice as high an isotope concentration as the entire molecule, indicating that C^{13} was present at alternate positions, i.e., at the odd-numbered carbon atoms, one of which is the carbon of the carboxyl group. Additional support for the view that the acetic acid carbon was distributed in random fashion over the entire fatty acid chain is afforded by analysis of the degradation products of the "oleic acid" fraction of tissue fat. This consists mainly of oleic acid and some palmitoleic acid and yields on oxidation a monocarboxylic acid, pelargonic acid, derived from carbon atoms 10 to 18 and a dicarboxylic acid (azelaic acid) representing carbon atoms 1 to 9. If a C_{18} fatty acid were formed from nine acetate units then of the nine isotopic carbons, five should be present in the azelaic acid and four in the pelargonic acid fraction, while the deuterium content of the two moieties should be nearly equal. The following isotope concentrations were found: azelaic acid, 0.090 per cent C^{13}, 0.14 per cent D; pelargonic acid, 0.071 per cent C^{13}, 0.15 per cent D. These data suggest a process of fatty acid synthesis involving the multiple condensation of C_2 units. Any scheme proposed as a mechanism of fatty acid synthesis must account for the fact that the fatty acids of animal tissues have an even number of carbon atoms and comprise, if milk fat is included, all members of the series from C_4 to C_{24}. The occurrence of C_2 condensation in the synthesis of fatty acids by animal tissues was first demonstrated by the finding of Stetten and Schoenheimer (130) that deuteriostearic acid is formed from deuteriopalmitic acid by chain elongation.

The reactive two carbon compound undergoing the condensation can be readily formed from acetic acid in the intact animal and in bacterial systems, but its chemical identity has not been determined. Fatty acid synthesis in animal cells has so far not been demonstrated in isolated systems. When rat liver slices were incubated with labeled acetic acid only very small amounts of isotope were incorporated into the fatty acids; significant concentrations of deuterium were found in the liver fatty acids when the buffer medium contained D_2O (131). From this result it cannot be decided whether the uptake of isotope resulted from synthesis of the carbon chain or whether it was due to hydrogenation of unsaturated fatty acids.

The production of C_2 units in the course of fatty acid oxidation and their utilization in fatty acid formation points to a mechanism of synthesis which consists of the reversal of the catabolic steps. This would involve the condensation of two carbon units with acetate and higher fatty acids and the subsequent reduction of β keto compounds to saturated acids. The well established conversion of acetate to acetoacetate may represent the initial step in this process. However, the biological reduction of β-keto acids to the corresponding saturated fatty acids has never been shown to occur.

Experiments with acetic acid containing deuterium as well as C^{13} have thrown some light on the nature of the intermediates involved in fatty acid synthesis (117). When a preparation of acetate was fed in which deuterium and carbon

were present in a ratio of 8:1 the fatty acids isolated contained the two isotopes in a ratio of 2.5:1. Irrespective of the nature of the condensation, there should be introduced for every two deuterium atoms from the methyl group of acetic a minimum of two atoms of ordinary hydrogen from the body fluids. The ratio of $D:C^{13}$ should then become 4:1. The greater loss of D than corresponds to this ratio may have resulted from keto-enol tautomerism of the intermediate β-keto acid. An additional atom of normal hydrogen from the body fluids would also be introduced if the keto acid were converted to the saturated acid by way of the hydroxy- and unsaturated acids.

Although the isotope data suggest that acetic acid can supply the carbon atoms for all positions of the fatty acid chain, it is unlikely that acetate is a precursor in the sense that its administration produces a net increase of synthesized fat. Experimental conditions for studying fat synthesis from specific precursors, similar to those by which the glycogenic or ketogenic properties of a compound can be tested, are not available. Conditions which result in the deposition of excessive quantities of fat in the liver such as choline deficiency do not measure changes in the amount of fat synthesized but reflect deficiencies in fat transport. Stetten and Boxer found that in rats treated with alloxan, fat deposition and incorporation of deuterium from heavy body water are greatly reduced (132). This may be the result of diminished fat synthesis or of a failure to deposit the newly formed fat. The impairment of fatty acid synthesis in this condition is probably not due to a lack of precursor but may reflect, as Stetten and Boxer suggest, a general inability to utilize carbohydrate. Since in the experiment of Rittenberg and Bloch (117) the animals received a fat free diet, carbohydrate and protein must have provided the carbon for fatty acid synthesis. It was estimated that under these conditions about 25 per cent of the carbon atoms of the fatty acids were derived from acetate. While the conversion of carbohydrate to fat is well established from balance experiments, no information is available concerning the intermediate steps. It is generally assumed that synthesis of the fatty acid chains occurs with pyruvate or a two carbon compound derived from pyruvate. It is questionable whether this intermediate is identical with acetic acid because in this case not only one-fourth but all carbon atoms of the fatty acids should be available from the "acetyl" pool. It has been pointed out above that pyruvic acid or alanine are not convertible to the acetyl compound involved in the acetylation of sulfanilamide or in the formation of cholesterol. This has been taken to mean that a direct degradation of pyruvic acid to a C_2 compound does not occur or that the product is not identical with the acetyl formed by fatty acid oxidaton or from acetate itself.[3] In order to allow for the participation of both acetic acid and carbohydrate in fatty acid synthesis, Rittenberg and Bloch (117) have suggested that pyruvate may form acylpyruvic acids and that the decarboxylation to β-keto acids takes place after the condensation

[3] This conclusion was based on experiments with deuterio alanine. It does not take into account the possibility that pyruvate is converted to acetyl by a circuitous route in which deuterium is lost entirely. Recent experiments of Sonne et al. (98) on uric acid synthesis with test substances containing C^{13} similarly indicate that lactate is not converted to acetate.

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$$CH_3COOH + CH_3COCOOH \rightarrow RCH_2COCH_2COCOOH \rightarrow RCH_2COCH_2COOH + CO_2 \quad [9]$$

In this reaction the net effect is the addition of a two carbon unit, without acetic acid appearing as an intermediate. It is also conceivable that an intermediate of the tricarboxylic acid cycle which contains the elements of both acetic and pyruvic acids supplies the C_2 fragments for fatty acid synthesis. For instance, the C_4 dicarboxylic acids which are derived from the condensation product of acetate and oxaloacetate are composed of two C_2 moieties one of which originates from acetate and the other from pyruvate. By passing through a symmetrical stage, randomization would result, and in a utilization of the dicarboxylic acid for fatty acid synthesis, an equal chance would exist that the C_2 fragment was contributed by either acetate or pyruvate.

It has been pointed out above that the acetic acid arising in intermediary metabolism appears to be derived primarily from the catabolism of fatty acids and ketogenic amino acids while carbohydrate does not contribute significantly to the acetate pool. It would follow, then, that the acetic acid employed for fatty acid synthesis has its origin in the fatty acids themselves; in other words, the process of acetate utilization merely reflects the continuous molecular regeneration of body fats. Since a large portion of the acetyl fragments arising in fatty acid oxidation is metabolized further and is therefore unavailable for fat resynthesis, carbon has to be provided from another source in order to keep the total quantity of fat constant. The source is evidently carbohydrate. Acetate is therefore not a precursor of fatty acids in the conventional sense and the statement that 25 per cent of the carbon atoms are supplied by acetate merely implies that one out of every four carbon atoms incorporated into a newly synthesized fatty acid molecule arose from the catabolism of the fatty acids themselves.

Cholesterol. In their experiments on the utilization of deuterioacetate by yeast Sonderhoff and Thomas found the deuterium concentration in the unsaponifiable fraction to be twice as high as in the fatty acid fraction and twenty times that of the yeast carbohydrate (127); they therefore suggested that in the synthesis of the yeast steroids acetate had been employed directly. A formation of cholesterol in animal tissues by total synthesis from small units was indicated by the findings of Rittenberg and Schoenheimer (112). In cholesterol newly formed by animals whose body fluids were enriched with heavy water roughly half of the hydrogen atoms were derived from the D_2O of the body fluids. The utilization of various small molecular substances for cholesterol synthesis has been investigated by Bloch and Rittenberg in feeding experiments with labeled compounds (133, 118, 78). The isotope concentration in cholesterol isolated from the tissue lipids was highest when labeled acetic acid was the test substance. Whenever another compound was effective in causing the formation of labeled cholesterol the result could be attributed to intermediary formation of acetate from the test substance (butyrate, ethanol, leucine, valerate, isovalerate). By studying simultaneously cholesterol synthesis and the acetylation of foreign amines it could be shown that the ability of a test substance to cause the formation of

labeled cholesterol was roughly parallel with its ability to furnish labeled acetyl groups. This correlation did not exist in the case of alanine. The significance of this finding has been discussed above. An evaluation of the quantitative rôle of acetic acid as a precursor for the steroid structure led to the conclusion that about half of the hydrogen atoms (and probably half of the carbon atoms) are furnished by acetate. Acetic acid appears to contribute to the formation of the entire steroid molecule. When cholesterol formed biologically from deuterio acetate was degraded, the iso-octyl fragment derived from the cholesterol side chain and the hydrocarbon representing the steroid skeleton contained deuterium in nearly equal concentrations. No information is available as to the intermediate steps of the synthetic process. The higher fatty acids or intermediates of fatty acid metabolism cannot lie on the path of the acetate-sterol conversion, since in feeding experiments with deuterio acetate, newly formed cholesterol contains several times the isotope concentration of the fatty acids; also, in liver slices deuterium is rapidly incorporated into cholesterol when labeled acetate is present, but not into fatty acids (131). The utilization of both carbon atoms of acetic acid is evident from the appearance of both deuterium and C^{13} in cholesterol synthesized from $CD_3C^{13}OOH$ (117, 131).

Since the acetate-cholesterol conversion entails essentially the transformation of—$CD_2 \cdot CO$—into—$CD_2 \cdot CH_2$—two normal hydrogen atoms should be incorporated for every two carbon-bound deuterium atoms and in the utilization of $CD_2C^{13}OOH$ the ratio of D to C^{13} should decline to half its original value. However, the relative amounts of D and C^{13} introduced into cholesterol were roughly the same as in the administered acetate and it would appear therefore that the reaction product contained, perhaps as the result of decarboxylations, more of the methyl carbons than of the carboxyl carbons of acetic acid.[4]

Some indications exist that a reduction may be the initial step in the acetate-cholesterol conversion. McLean and Hoffert (126) observed that sterol synthesis from acetate in yeast was inhibited by sulfite, a reagent which blocks aldehyde groups. In the formation of labeled cholesterol by rat liver slices, labeled ethanol and acetaldehyde were somewhat more effective than acetic acid (97) suggesting that the intermediate is more readily formed from ethanol and acetaldehyde. It is unlikely that the somewhat higher efficiency of acetaldehyde and ethanol under these conditons is due to the fact that they diffuse more readily into the liver cell since the deuterium concentration in cholesterol is independent of the acetate concentration in the buffer medium over a wide range (131).

In the intact animal and in liver slices deuterio alanine fails to give labeled cholesterol (118, 97). Alanine, and presumably pyruvic acid, are therefore not convertible to the C_2 compound which provides carbon for cholesterol synthesis and which is readily formed from acetic acid itself and from the higher

[4] The results obtained with intact animals differ from those obtained in liver slices. The ratio of D to C^{13} in cholesterol formed from acetate in vitro changes from 8 to 2.5. This might be explained by the loss of carbon bound deuterium associated with the interconversion of acetate and acetoacetate which proceeds rapidly in liver slices but apparently not in the intact animal.

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fatty acids. This finding supports the previously expressed view that if a C_2 fragment arises in carbohydrate oxidation it is unlikely to be identical with that derived from fatty acids.

The origin of the remaining carbon and hydrogen atoms in cholesterol, which are not accounted for by acetic acid, is unknown. In view of the fact that cholesterol contains a branched side chain and angular methyl groups and that these groupings are otherwise present only in dietary constituents which animals cannot synthesize (valine, leucine, carotene), the possibility has been tested that one of the branched chain amino acids participates in steroid synthesis. The feeding of leucine which contained deuterium at all positions of the carbon chain resulted in the formation of labeled cholesterol while similary labeled valine was ineffective (78). However, leucine is a ketogenic amino acid and in the course of its catabolism yields acetyl groups. Hence, no conclusions may be drawn as to whether leucine specifically supplies the branched side chain and the angular methyl groups of cholesterol or whether the observed effect is ascribable merely to the intermediary formation of acetic acid.

Cholesterol can serve as the parent substance for other compounds which possess the cyclopentano-phenanthrene structure. The in vivo transformation of cholesterol into cholic acid (134) and into pregnanediol (135) has been demonstrated with the aid of deuteriocholesterol and it has been found that a minimum of two-thirds of these steroids can arise by degradation of cholesterol. It is therefore not too likely that animal steroids other than cholesterol can also be formed by total synthesis from small units by a pathway in which cholesterol is not an intermediate.

Protoporphyrin. Hemin isolated from the red cells of animals which receive deuterio acetic acid contains deuterium in significant concentrations (30). In short term feeding experiments the isotope content of hemin will attain only a relatively low level, since the protoporphyrin moiety of hemoglobin is synthesized at a much slower rate than most body constituents (114). When deuterioacetate feeding is extended to a period of several weeks the deuterium concentration in hemin reaches a value corresponding to 50 per cent of the isotope concentration in the acetic acid available in the metabolic pool (136). Therefore at least half of the hydrogen atoms in the porphyrin molecule are supplied by acetic acid. The constituent pyrrol nuclei in the porphyrin molecule contain no hydrogen linked to carbon; carbon bound hydrogen is present in the methin bridges which link the pyrrol rings and in the pyrrol side chains. Hence, experiments with deuterio acetic acid do not reveal whether acetic acid is also a source of carbon atoms for the pyrrol rings. A mechanism of synthesis which leads directly to a pyrrol structure containing the appropriate side chains would seem to be a more likely event than a process in which the substituent side chains are attached to a preformed pyrrol nucleus. No deuterium is introduced into hemin after the feeding of deuterio propionic acid while β-γ-dideuterio butyric acid is about half as effective as deuterio acetic acid (136). The effect shown by butyrate is most likely due to its splitting into acetyl fragments.

The origin of pyrrol nitrogen has been elucidated by the experiments of

Shemin and Rittenberg (114, 137). Hemin isolated from the blood of a human subject who had received glycine labeled by N^{15} contained so high an isotope concentration as to indicate a specific utilization of glycine nitrogen. The specific role of glycine in pyrrol synthesis received further support from the finding that glycine was the most effective source of pyrrol nitrogen among several amino acids tested (114). In particular, proline and glutamic acid, which have been suggested as possible biological precursors of pyrrols because they contain or can readily form heterocycles similar to pyrrols, were much less effective as sources of nitrogen for hemin than glycine. The utilization of the two carbon compounds, acetic acid and glycine, points to a mechanism of porphyrin synthesis in which the ring structure and the substituents are formed simultaneously, possibly by processes analogous to those which yield pyrrols in organic chemistry. Fischer and Fink (138) have obtained evidence that ring closure to a pyrrol occurs when glycine and formylacetone (the aldehyde of acetoacetic acid) react in aqueous solution at 37°. The classical pyrrol synthesis of Knorr (139) involved the condensation of α-amino acetoacetic ester with acetylacetone. Acetic acid can be converted to aceto-acetate in vivo and it is not inconceivable that by an analogous process α amino-acetoacetic acid is formed biologically from acetic acid and glycine. A biological pyrrol synthesis analogous to the Knorr reaction would be attractive because it would afford a product in which the arrangement and the chemical nature of the substituents resembles that in the naturally occurring protoporphyrin.

Uric acid. Recently the utilization of C_2 compounds for the synthesis of still another group of tissue constituents has been reported. Sonne, Buchanan and Delluva (98) have studied the incorporation of C^{13} into uric acid, isolated from pigeon excreta, after the feeding of $CH_3C^{13}OOH$, $CH_3^{13}C^{13}HOHCOOH$, $CH_3CHOHC^{13}OOH$ and $NH_2CH_2C^{13}OOH$ respectively. The carboxyl carbon of acetic acid was located at the two ureido carbons (2 and 8) of the purine structure, while the carboxyl group of glycine supplied carbon for position 4, and CO_2 carbon for position 6. On the basis of the data available so far no plausible mechanism for purine synthesis from CO_2, acetate and glycine can be suggested. It is of interest in this connection that Barker and Beck (140) found ammonia, carbon dioxide, acetic acid and small amounts of glycine to be formed as products of uric acid fermentation by *Clostridium acidi urici*. The findings of Sonne et al. contain important evidence with respect to some other metabolic processes. The appearance of acetate and glycine carbon at different positions of the uric acid eliminates the possibility that in the pigeon glycine and acetic acid are interconvertible. On the basis of nutritional studies, Almquist et al. had contended that the chick is unable to synthesize adequate amounts of glycine and that in deficient diets acetate may serve as substitute for glycine (141). The interconvertibility of the two compounds which is implied by these findings is contradicted by the tracer experiments of Sonne et al. unless metabolic differences exist between the chick and the pigeon.

The isotope data obtained by Sonne et al. furthermore show that the α carbon of lactate is not incorporated into the same positions of uric acid as the carboxyl

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carbon of ac[illegible] A breakdown of lactate to acetate did, therefore, not occur to any considerable extent. These results are incompatible with the assumption that the hypothetical two carbon compound arising in pyruvate oxidation is closely related to acetic acid.

A relationship between acetate and lipid metabolism in bacteria has been observed by Guinard, Snell and Williams (142). Acetate stimulates the early growth of lactic acid bacteria, an effect which is also shown by fatty acids and various steroids. These results suggest an important rôle of acetate in the synthesis of lipids by bacterial systems.

As the result of investigations with isotopically labeled test substances there have been developed a number of novel biochemical theories among which Schoenheimer's concept of the dynamic state of the body constituents has been the most outstanding (143). According to this concept, there exists a pool of metabolites which is continuously replenished by the breakdown products of both dietary and tissue constituents and which supplies the metabolites for energy production and for the rebuilding of the structural components of the cell. The concept of the metabolic pool was advanced in order to interpret the data obtained from feeding experiments with isotopic substances and in order to provide a reasonable basis for the rapid interchange of dietary and tissue elements which was indicated by these results. It should be pointed out that the metabolic pool may not possess any physical reality in the sense that there exists in the body at a given moment a large reservoir of metabolites in which molecules derived from dietary and various tissue constituents are distributed in random fashion and are therefore undistinguishable. It is merely permissible to say that if for example 100 molecules of dietary origin enter the tissues during a given period of time and 100 molecules arise from body constituents during the same interval, then the chances that in a subsequent reaction either the exogenous or endogenous molecule is employed will be equal. The metabolic pool should therefore be regarded merely as an expression of this probability.

One of the problems of metabolism which has held the general interest of the biochemist has been the mutual interconversion of fat, carbohydrate and protein. These questions have only partially been answered by balance techniques in which the criterion of a biochemical conversion is the net change in amount of the reaction product which is induced by the precursor. In the light of the concept of the metabolic pool, biochemical interconversions assume a different meaning. Fat, protein and carbohydrate are interconvertible in the sense that they give rise to breakdown products which lose the identity of their origin and can be utilized interchangeably for the resynthesis of either one of the three major tissue constituents.

The fact that acetic acid is employed as a building stone in the synthesis of a variety of compounds of different chemical structure and function, viz. lipids, carbohydrate, porphyrin, uric acid and some of the amino acids, suggests its direct or indirect participation in the biological formation of all compounds which can be synthesized by the animal cell. If this is true then acetic acid cannot be regarded as a specific precursor of a particular tissue constituent, but its utilization would reflect merely the fact that the body employs two carbon units as a principal source for synthetic reactions.

The biologically active form of acetic acid. Since in isolated tissues acetic acid shows less reactivity than in the intact animal the possibility has been widely discussed that acetic acid is metabolically converted into a more reactive form. In organic chemistry such activations are achieved by esterification or anhydride formation (acetylchloride, acetic anhydride, ketene, ethylacetate).[5]

Experimental evidence for the biological formation and the participation in a synthetic reaction of an acetic acid derivative rather than of acetic acid itself has been provided in two instances. In bacterial systems the phosphoroclastic splitting of pyruvate to acetylphosphate and formate can be reversed (145). No other function for acetylphosphate has been found so far. A process of intermolecular acetyl transfer in which acetyl groups are shifted from an N-acetyl amino acid to a free amino acid has been indicated by acetylation studies in the intact animal (35).

There are two other well known biochemical processes which are classified as transfer reactions, transfer of methyl groups involving choline, methionine and creatine (146) and the amidine transfer from arginine to glycine in the formation of guanido acetic acid (147, 148). Such reactions cannot be described in terms of ordinary chemical equations involving stable compounds as intermediates. It is conceivable that group or radical transfer may be a more general phenomenon in biological systems and that the answer to the acetyl problem may be sought in this direction.

THE OXIDATIVE METABOLISM OF ACETIC ACID. The ability of animal tissues to utilize acetic acid efficiently is illustrated by the fact that when fed to animals acetic acid does not appear in the urine in measurable amounts. In the experiments of Buchanan, Hastings and Nesbett with acetate containing radioactive carbon in the carboxyl group, over fifty per cent of the acetate absorbed by the rats was totally oxidized within two hours, as determined by the appearance of isotopic carbon in the expired carbon dioxide (120). This rate was comparable to that at which similar quantities of lactic acid were metabolized (149).

Acetate disappears aerobically from a variety of isolated tissues, the rate being most rapid in the cortex of guinea pig-kidney (150). Little evidence exists that the acetate molecule as such can undergo oxidative reactions.[6] Products which could conceivably arise by oxidation of the methyl group of acetic acid such

[5] For a consideration of the "active" form of acetic acid the following organic chemical reactions are of interest. The studies by Bergmann et al. (36) on intermolecular transfer of acetyl groups or radicals between amino compounds have been mentioned above. Kharasch and Gladstone (144) have shown that by conversion into diacetyl peroxide, the methyl groups of acetic acid become sufficiently activated to undergo α-α condensation to succinic acid. The reaction is believed to be due to the dimerization of an intermediate CH_2COOH radical.

[6] The claim of Toenniessen and Brinkmann (151) that formate is a product of acetate metabolism in perfused muscle is based on insufficient experimental evidence, but conforms with recent observations of Buchanan and Sonne (167) on the rôle of acetate and formate respectively as precursors of uric acid.

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as glycolic, glyoxylic, oxalic acids or glycine are metabolically inert under conditions which permit of rapid oxidation of acetate (152). Many years ago Thunberg suggested that the oxidation of acetate was initiated by the dehydrogenative coupling of the methyl groups of two molecules of acetate to succinic acid (55).

$$HOOC\cdot CH_2\boxed{H + H}CH_2COOH \longrightarrow HOOC\cdot CH_2CH_2COOH \quad [10]$$

The same reaction was proposed later by Wieland and Sonderhoff to account for the formation of succinate by yeast in which acetate was the sole nutrient (153). Slade and Werkmann (108) have studied this process with C^{13} containing acetate in *Aerobacter indologenes* and have concluded that the isotope data demonstrate methyl condensation of two moles of acetate. Lipmann has suggested that this condensation may involve acetylphosphate (154). The inhibition by malonate of acetate and acetoacetate disappearance in animal tissues (155, 156) and of acetate in yeast (157) has been cited in support of reaction [10] but the fact that acetate oxidation is sensitive to malonate may merely be taken to suggest that succinic acid lies on the path of acetate metabolism; it does not indicate the mechanism involved. There is increasing evidence that the step which precedes acetate oxidation consists of a condensation with a C_4 dicarboxylic acid. From the resultant C_6 tricarboxylic acid two carbon atoms are removed by oxidation to regenerate the dicarboxylic acid:

$$CH_3COOH + C_4 \rightarrow C_6 \rightarrow 2\,CO_2 + C_4 \quad [11]$$

The experimental support for this scheme coincides in many instances with the evidence which led originally to the formulation of the "citric acid" cycle for the metabolism of pyruvic acid. In fact, in some respects the rôle of the tricarboxylic acids is better understood for the oxidative phase of fatty acid and acetate metabolism than for the oxidation of pyruvate. Acetate and acetoacetate have been shown to form the intermediate polycarboxylic acids of the cycle, but it has remained undetermined whether pyruvate itself enters into combination with the C_4 dicarboxylic acids or whether the condensation is preceded by a degradation of pyruvate to a two carbon unit. The significance of the citric acid cycle in the metabolism of fat and carbohydrate respectively is still a matter of controversy. According to Breusch, carbohydrate is not oxidized to an appreciable extent by way of the citric acid cycle (158), while Weil-Malherbe (159) and Krebs (160) reject the view that the citric acid cycle is involved in the oxidation of acetoacetate.

Acetoacetate, acetate and pyruvate have been shown to be convertible, in the presence of dicarboxylic acids, to citrate and intermediates of the tricarboxylic acid cycle, but whether a single mechanism is invoved in all cases is undecided. However, the citric acid cycle constitutes the most successful attempt to date to describe the oxidative phases of fat, carbohydrate and protein metabolism by a unified scheme which visualizes a junction of metabolic pathways and leads to a common mechanism for the ultimate oxidation of the three major dietary and tissue constituents.

The citric acid cycle in relation to the metabolism of carbohydrate has been discussed in reviews by Werkmann and Wood (161), Krebs (162) and Evans (163). The citric acid cycle as a vehicle for fat oxidation has been considered in detail by Wood (123). The present discussion will be concerned primarily with experimental data which deal with the oxidation of acetic acid.

Since there is little evidence to support the assumption that the carbon chain of acetate as such is susceptible to oxidation the question arises as to the steps which precede the conversion of acetate to carbon dioxide and water. Breusch (158) has suggested that the intermediate β keto acids formed in fatty acid oxidation combine directly with oxalacetate to give a product which contains the two terminal carbon atoms of the fatty acid chain, leaving a fatty acid shortened by two carbon atoms. According to this view, two carbon compounds would not arise as distinct intermediates in fat oxidation. Since acetic acid is a compound of great chemical stability and, compared to other metabolites, relatively unreactive in some biological systems, the view has frequently been expressed (123) (164) that acetic acid itself may never arise but may be the stabilization product of a more reactive C_2 unit. The identity of this intermediate has not been established in animal tissues. Judging from the variety of reactions which it undergoes in the intact animal and in some isolated systems, acetic acid must be readily convertible to the hypothetical reactive C_2 fragment.

$C_2 + C_1$ addition. The reversal of the phosphoroclastic splitting of pyruvic acid to formate and acetylphosphate according to the equation:

$$CH_3COCOOH + H_3PO_4 \rightleftarrows CH_3COOPO_3H_2 + HCOOH(H_2 + CO_2) \quad [12]$$

is well established in bacterial systems (145). Lipmann has demonstrated convincingly that acetyl phosphate is the product of this bacterial dissimilation (165). The preservation of a large fraction of energy in the carboxylphosphate facilitates the reverse reaction, i.e., the carboxylation of the C_2 compound, a reaction which is energetically improbable with acetate itself. It has not been possible to demonstrate acetylphosphate as a product of pyruvate metabolism in animal tissues nor can the evidence for the formation of acetylphosphate from acetate in animal tissues be considered sufficient. Lipmann and Tuttle (166) have incubated extracts from pigeon liver with acetate and adenosine triphosphate and observed that on addition of hydroxylamine and ferric chloride a chromogen was formed which had the properties of hydroxamic acids obtainable from acetylphosphate or aldehydes, but more direct proof is desirable to establish the identity of the acetyl compound formed under these conditions. The reversal of reaction [12] is a formylation rather than a carboxylation and apparently CO_2 can be utilized only in organisms which hydrogenate carbon dioxide to formate. In considering the likelihood that reaction [12] occurs in animal tissues, it should be noted that so far no function has been assigned to formic acid in animal metabolism.[7] Experiments of Lorber et al. (121) in which the isotope distribu-

[7] This statement may have to be revised in view of the recent finding by Buchanan and Sonne (167) that formate is utilized in uric acid synthesis by pigeons. The authors suggest that formic acid may arise by oxidation of acetate.

tion in glucose was studied after feeding of labelled acetic acid contain no evidence for $C_2 + C_1$ addition. Carboxylation of acetic acid which contained C^{13} in the carboxyl group would yield pyruvate with the labeled carbon at the α carbon atom. If such pyruvate were further carboxylated to oxaloacetate and reformed after passing through the stage of a symmetrical dicarboxylic acid, the isotopic carbon would become equalized between the α and β positions of pyruvate. Glucose formed from the singly labeled pyruvate should contain isotope at positions 2 and 5 and, if formed from doubly labeled pyruvate, in positions 1 and 6 as well. Actually a significant excess of C^{13} was encountered only at positions 3 and 4. Thus a carboxylation of acetate to pyruvate cannot be responsible for the incorporation of acetate carbon into the glucose units of glycogen. Data pertaining to the formation of glycogen or intermediates of the citric acid cycle in the presence of labeled CO_2 do not permit any deductions as to the occurrence of acetate carboxylation because any labeled carbon entering by the latter reaction becomes indistinguishable from the carbon which is incorporated into the C_4 dicarboxylic acids by the primary carboxylation of pyruvate to oxalacetate.

$C_2 + C_2$ addition. There exist several observations which are consistent with Thunberg's suggestion (55) that succinate might arise by the linking of the methyl groups of two moles of acetate. The conversion of acetate to succinate by yeast with acetate as the sole nutrient was attributed by Wieland and Sonderhoff to the dehydrogenative coupling of acetate (153). In an analogous experiment with deuterio acetate Sonderhoff and Thomas (127) found high concentrations of deuterium in succinate and citrate and interpreted their results as evidence for reaction [10]. The proof for this contention would be conclusive only if the succinate contained close to four atoms of deuterium instead of the two atoms actually found. More than one pathway can account for the observed level of isotope in succinic acid. The same qualification applies to the findings on succinate formation from labeled carbon containing acetate by *Aerobacter indologenes* (168). The possibility that in both cases succinate arose by way of the tricarboxylic acid cycle cannot be excluded. At the present moment no experiments are available which establish the occurrence of reaction [10] in the conversion of acetate to succinate. Whenever succinate formation from acetate is demonstrable, succinate is not the sole product but α-ketoglutarate and tricarboxylic acids also accumulate. If acetate were metabolized according to equation [10] a mechanism should also be available for the subsequent conversion of succinate to ketoglutarate and citrate. The discovery of the carboxylation of α-ketoglutarate to oxalosuccinate by Ochoa (168) provides a pathway to the tricarboxylic acids, but the reversibility of the oxidative decarboxylation of ketoglutarate to succinate remains to be demonstrated.

$C_2 + C_3$ addition. The possibilities have been considered that acetic and pyruvic acids combine to form the five carbon acids α-ketoglutaric and aceto-pyruvic acids. As a result of the current prominence of the tricarboxylic acid cycle these two processes, which would lead directly to five carbon acids, have received diminishing attention. Krebs and Johnson (169) have suggested the formation of aceto-pyruvate according to the equation

$$CH_3CO\boxed{OH + H}CH_2COCOOH \rightarrow CH_3COCH_2COCOOH$$
$$CH_3COCH_2COCOOH + \tfrac{1}{2}O_2 \rightarrow CH_3COCH_2COOH + CO_2 + H_2O \qquad [13]$$

as a conceivable intermediate for the conversion of pyruvate to acetoacetate in liver. The utilization of acetopyruvate by intact animals and in isolated tissues has been studied by Lehninger (170). The compound was found to be ketogenic. Since acetoacetate can be metabolized by way of the tricarboxylic acid cycle the reaction offers a chemically plausible scheme for the conversion of pyruvate to the oxidation level of acetate.

Acetopyruvate cannot be an obligatory intermediate in ketone body formation from fatty acids. Weinhouse et al. (76), in a study of the oxidation of labeled octanoic acid by liver, found an isotope distribution in acetoacetate which eliminated the participation of pyruvate as an essential component. It is however conceivable that ketone bodies can be formed by two independent synthetic processes, by condensation of two molecules of acetate and by combination of acetate with pyruvate.

α-Ketoglutarate is a key substance in the tricarboxylic acid cycle and a demonstrable intermediate whenever fatty acids or pyruvate are oxidized. A process leading to α-ketoglutarate by "end to end" condensation of acetate and pyruvate has been suggested by Krebs (171):

$$HOOCCH_2\boxed{H + H}CH_2COCOOH \rightarrow HOOCCH_2CH_2COCOOH \qquad [14]$$

Weinhouse et al. have observed the formation of labeled α-ketoglutarate from isotopic acetate and normal oxaloacetate in rat kidney slices or mince, but found no evidence for the simultaneous formation of marked tricarboxylic acids (172). In their experiments non-isotopic citrate and cis-aconitate were added as carriers to facilitate the recovery of citrate at the end of the incubation period. The failure of acetate carbon to become incorporated into citrate in spite of the presence of aconitase, led the authors to the conclusion that citric acid as such was not in the pathway of acetate oxidation by kidney. These results would seem to suggest that α-ketoglutarate arose from acetate and pyruvate according to equation [14] without intervention of the tricarboxylic acids. However in this case the data exclude rigidly only the formation of citric acid itself. It does not appear to be feasible to isolate isocitric acid or cis-aconitate from such experiments. That the latter two tricarboxylic acids may have been intermediates becomes clear from kinetic data of Martius (173) and Krebs (174). These authors have shown that in systems containing the tricarboxylic acids and aconitase the conversion of cis-aconitate to isocitrate proceeds much more rapidly than to citrate. As the conversion of isocitrate to α-ketoglutarate is also a rapid reaction it is possible that in the experiments of Weinhouse et al. cis-aconitate or isocitrate were the initial reaction products but that the breakdown to α-ketoglutarate proceeded at a much faster rate than isomerization to citrate.

In a more recent investigation (175) the same authors report that after incubation of kidney with isotopic acetoacetate and normal oxaloacetate, without addition of non-isotopic carrier, citrate could be isolated directly and shown to

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contain an isotope level which indicated that about two-thirds of the citrate arose from the isotopic acetoacetate. These recent experiments differed from earlier ones only in that acetoacetate instead of acetate was the substrate. In view of the fact that acetate is readily convertible to acetoacetate under these conditions it is not apparent why labeled citrate should not have formed in both instances.

In an analysis of the available data pertaining to the citric acid cycle, Wood (123) points out that α-ketoglutarate formation according to reaction [14] as opposed to its formation from tricarboxylic acids cannot be ruled out at the present. If reaction [14] were responsible for α-ketoglutarate formation rather than the oxidative decarboxylation of isocitric acid, then the formation of tricarboxylic acids would have to be ascribed to the carboxylation of α-ketoglutarate to oxalosuccinate and reduction of the latter to isocitrate. Experiments with labeled test substances and studies of isotope distribution in the α-ketoglutarate formed from either labeled acetate or acetoacetate have been incapable of providing an answer as to which reaction occurs. Labeled carbon will occupy the same positions whether α-ketoglutarate is formed by oxidative decarboxylation of isocitrate, directly from acetate and pyruvate or by carboxylation of succinate formed according to equation [10]. The condensation of oxaloacetate and acetate to a tricarboxylic acid does not as such involve an oxidation but can be formulated either as an addition if isocitrate is the primary product, or as a condensation with elimination of water if cis-aconitate is initially formed. It differs in this respect from reactions [10] and [14] in which the coupling process is oxidative. Experimentally it has been found that the formation of the intermediates of the citric acid cycle requires aerobiosis (176) unless a hydrogen acceptor such as oxaloacetate is present in excess (177). This may indicate the occurrence of oxidative condensations or, as is generally believed, oxygen may be necessary to effect the transformation of the reactants into activated forms or derivatives.

There is only scant information on the mechanisms by which carbon-carbon bonds are established biologically. It may be pointed out that in the few processes in which the chemical identity of the reacting molecules seems well established, e.g., in the formation of hexose from triose, or of acetoacetate from acetate, or in the carboxylation of pyruvate to oxaloacetate, at least one of the reacting groups is a carbonyl group. No instances are known of a formation of carbon to carbon bonds by elimination of hydrogen between methyl- or methylene groups.

$C_2 + C_4$ condensation. At present the ultimate oxidation of the split products of fatty acids can be explained most satisfactorily by assuming the intermediate formation of a tricarboxylic acid according to equation [11] and the subsequent breakdown of the condensation product by way of α-ketoglutaric acid and the C_4 dicarboxylic acids. Reaction [11] was originally proposed by Virtanen (178) to account for citrate formation from acetate in micro-organisms. The following evidence has been secured in support of the suggested rôle of the citric acid cycle for fat and acetate oxidation in various biological systems. Wieland and Sonderhoff (153) found succinate and citrate to accumulate as products of acetate

oxidation by yeast when acetate was the sole nutrient. Under these conditions, the yield of citrate is increased by the addition of oxaloacetate (179). Succinate and citrate formed from deuterioacetate contained 50 per cent and 46 per cent respectively of the deuterium concentration of acetate in stably bound form (127). Lynen has contributed important evidence to show that the formation of succinate from acetate in yeast involves the tricarboxylic acid cycle (157). He demonstrated that the malonate inhibition of acetate disappearance could be overcome by fumarate or oxalacetate and cites this finding as evidence for a requisite participation of the C_4 dicarboxylic acids in acetate oxidation. The observation that the rapid disappearance of acetic acid in yeast is preceded by an induction period was interpreted to show that a second component must accumulate before acetate can be metabolized at the maximum rate. A stimulatory effect of citrate, α-ketoglutarate and the C_4 dicarboxylic acids has been observed for the aerobic disappearance of acetoacetate in kidney homogenates by Buchanan et al. (180). Wieland and Rosenthal (176) obtained optimum yields of citrate in kidney brei when acetoacetate was oxidized in the presence of oxaloacetate. The identity of the reaction product with citric acid was established by direct isolation. Hunter and Leloir (177) have shown that two molecules of extra citric acid arise from each mole of acetoacetate. They have made the observation that in their experimental system, which consisted of the insoluble particles of kidney cortex homogenate, the simultaneous oxidation of α-ketoglutarate was essential for citrate formation. This oxidative process is believed to furnish the necessary energy for the conversion of acetoacetate to the intermediate which condenses with oxaloacetate. Lehninger (89) has found that the oxidation of octanoic acid in a preparation of washed rat liver cells yields acetoacetate quantitatively, but that in the presence of fumarate, citrate and α-ketoglutarate accumulate in significant quantities at the expense of acetoacetate. According to Breusch (181) an enzyme can be extracted from kidney, brain and skeletal muscle which catalyzes the anaerobic condensation of β-keto acids with oxaloacetate to citrate.

In addition to the balance studies a number of experiments have been carried out with isotopically labeled substances. Buchanan et al. (180) incubated kidney homogenates with $CH_3C^{13}OOH$ or acetoacetate marked by heavy carbon at both carboxyl and carbonyl positions. Non-isotopic ketoglutaric acid, fumarate or succinate were added as carriers; when re-isolated after incubation these acids were shown to contain C^{13} in the carboxyl positions. It was calculated that the γ-carboxyl of α-ketoglutarate contained almost ten times as much C^{13} as the carboxyl carbon adjacent to the carbonyl group. Essentially the same findings were obtained by Weinhouse et al. (172). The formation of labeled citrate from acetoacetate recently demonstrated by the same authors (175) has been mentioned above.

Intermediates of the citric acid cycle can be obtained from isolated tissues on incubation with a variety of substrates but since they are short-lived intermediates *in vivo* they are not obtainable as such in sufficient amounts from intact animals. It has therefore not been possible to secure direct evidence as to

whether the tricarboxylic acid cycle is concerned in fatty acid oxidation also in the intact animal. Feeding experiments have been carried out by Buchanan, Hastings and Nesbett (129) with acetic, propionic and butyric acids, all of which contained C^{13} in the carboxyl group. Glycogen isolated from such animals contained radioactive carbon in concentrations which in the case of acetate feeding were attributable to incorporation of CO_2, but were significantly higher with radioactive propionate and butyrate. Evidently the latter two acids had furnished carbon for glycogen synthesis by conversion to intermediates of carbohydrate metabolism. With the aid of degradation procedures which allow isotope determinations at individual carbon atoms of the glucose molecule (115), Wood and his collaborators have been able to show that the carboxyl carbons of acetic acid appear at positions 3 and 4 (121) and the methyl carbons at positions 1, 2, 5 and 6 of the glucose molecule (182). Thus, both carbon atoms of acetic acid are involved in glycogen formation. Butyric acid $CH_3C^{13}H_2CH_2C^{13}OOH$ afforded the same isotope distribution in glycogen as $CH_3C^{13}OOH$ (182). From these findings the conclusion can be drawn that there exists a pathway for the conversion of the two carbon split products of fatty acids into the glucose units of glycogen. In this process each carbon atom of glucose can originate from fatty acid carbon.

Glutamic and aspartic acids are believed to be in biological equilibrium with α-ketoglutarate and oxaloacetic acid. Any incorporation of marked fatty acid carbon into intermediates of the tricarboxylic acid cycle should therefore become manifest by the formation of labeled aspartic and glutamic acids. The two amino acids have been isolated by Rittenberg and Bloch from the tissue proteins of mice which had received $CH_3C^{13}OOH$, and were shown to contain significant concentrations of C^{13} (183).

While the evidence which has accumulated in favor of the tricarboxylic acid cycle is impressive it should be emphasized that this scheme does not offer a unique explanation for the formation of tri- and dicarboxylic acids as products of fatty acid oxidation. The following points of the cycle need further clarification: 1, the identity of the intermediate of fatty acid and pyruvate metabolism which condenses with oxaloacetate; 2, the nature of the initial condensation product; 3, the existence of one or more mechanisms for the oxidation of acetate, acetoacetate and pyruvate respectively.

Although the stimulatory effect of the C_4 dicarboxylic acids on citrate formation is exhibited not only by oxaloacetate but also by succinate, malate and fumarate, it is reasonable to asume that the effect is attributable in all cases to oxaloacetate. The nature of the acetyl compound which participates in the condensation process is less evident. In some systems intermediates of the cycle are formed from acetate as well as from acetoacetate, while in other situations the two substrates cannot be employed interchangeably. In balance studies with kidney preparations (176, 177) and in the experiments of Lehninger (66) with a heart muscle preparation only acetoacetate was an effective precursor of citrate. The failure of acetate to react in kidney preparations is unexpected because acetate is readily converted to acetoacetate in this organ. On the other hand,

Buchanan et al. (180) found that labeled acetate was as effective as acetoacetate in forming labeled α-ketoglutarate in kidney homogenate. It should be reemphasized in this connection that with the aid of the tracer technique a conversion can be demonstrated even if there is no net change in the concentration of the reaction product, e.g., citrate or α-ketoglutarate. This is important in systems in which the product is metabolized further at a rapid rate.

In general, acetoacetate appears to be the more reactive molecule with regard to the condensation reaction. On the basis of their data Wieland and Rosenthal (176) postulated that acetoacetate itself condensed to yield the hypothetical procitric acids, citroylacetic or acetylcitric acids. Weinhouse et al. were able to isolate C^{13} containing citrate from kidney homogenate when acetoacetate was the substrate (175), while in earlier experiments under similar conditions, labeled acetate yielded isotopic α-ketoglutarate but not citrate (172). The preferential utilization of acetoacetate might be taken to indicate that acetate enters the tricarboxylic acid cycle by way of acetoacetate. However, several situations exist for which this explanation does not hold. Citrate is a product of acetate oxidation in yeast, but as far as is known, the conversion of acetate to acetoacetate does not occur in this organism. Medes et al. have shown that acetate undergoes complete oxidation in heart muscle but that acetoacetate is not an intermediate (86). In kidney also a portion of acetate was oxidized by a route which did not pass through the acetoacetate stage. The data allow for two possible explanations: 1, acetate and acetoacetate enter the tricarboxylic acid cycle by a different process, and 2, a reactive acetyl compound is formed from both acetate and acetoacetate before condensation occurs. In the utilization of acetoacetate a preliminary splitting to acetic acid can be excluded as an intermediate step. Buchanan et al. (184) added normal acetate and isotopic acetoacetate to an extract of rabbit kidney and found that the acetic acid recovered from the mixture after incubation contained appreciably less isotope than α-ketoglutarate or succinate. From energy considerations it is to be expected that an energy rich acetyl can arise far more readily from acetoacetate than from acetate. The inability of acetate to substitute for acetoacetate may be ascribed to an impairment of the enzyme system responsible for the conversion of acetate to the metabolically active form. Although acetoacetate is an efficient source of citrate in various tissue systems, it is highly doubtful that it is an obligatory intermediate in tricarboxylic acid formation from fatty acids. In the experiments of Lehninger with washed liver cells, acetoacetate, unlike octanoic acid, was incapable of yielding citrate (89). The preparation apparently had lost the property of converting acetoacetate into the active two carbon fragment. The reasons which led to the belief that in the intact animal acetoacetate is not an intermediate of fatty acid oxidation have been discussed above.

Since the experimental conditions employed by various investigators for the study of the same problem vary widely, particularly with respect to methods of tissue preparations, it is not surprising that such experiments have often yielded contradictory results and that it is not possible to fit all available data into a single scheme. It is evident that with increasing impairment of the cellular

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organi[illegible] the enzymatic complement of the biological system becomes increasingly deficient and hence the probability will diminish that a complex series of reactions can take place. Processes which have high energy demands and must be coupled with energy yielding reactions such as the conversion of acetate to an active acetyl are less likely to occur in homogenates or tissue extracts than in systems in which the cell structure is preserved.

Lipmann has shown that by the addition of adenosine triphosphate acetate can be activated to acetylate sulfanilamide anaerobically in pigeon liver extracts (2). The expectations, based on this finding, that the active C_2 compound which is involved in the tricarboxylic acid cycle may be identical with acetyl phosphate, have not been fulfilled. Under conditions in which citrate formation can be demonstrated to occur with acetoacetate but not from acetate, acetylphosphate is also ineffective (184, 177, 89). In order to determine the identity of the active two carbon compound, Lehninger (89) has also tested the effect of glycolate, glyoxalate, oxalate, acetaldehyde, ethanol; acetamide and glycine. None of the compounds yielded extra citrate under conditions which afforded citrate from octanoic acid.

Since cis-aconitate, isocitrate and citrate are biologically interconvertible, the identity of the tricarboxylic acid initially formed in the citric acid cycle has remained in doubt. In the presence of aconitase the system will contain all three acids irrespective of the nature of the primary condensation product (173, 185). The cycle as originally proposed by Krebs and Johnson (185), in which citric acid was the initial condensation product, had to be modified when it was found by Evans and Slotin (186) and by Wood et al. (187) that the α-ketoglutarate formed in pigeon liver homogenate from labelled CO_2 and pyruvate contained labeled carbon only in the carboxyl group adjacent to the carbonyl group, and that when malonate was present, the succinate formed contained little C^{13} (186). The tricarboxylic acids could therefore not have originated from a symmetrical citric acid. An unsymmetrical molecule must also have been the precursor of the dicarboxylic acids which Buchanan et al. (180) and Weinhouse et al. (175) obtained as the reaction product of labeled acetoacetate or acetate, and oxaloacetate. In the α ketoglutarate obtained from the latter experiments only one-sixth and one-fourth respectively of the total isotope was present in the carboxyl adjacent to the keto group; it should have contained half of the total, if a symmetrical tricarboxylic acid had been the precursor. Likewise, in glutamic acid isolated from the proteins of animals which had received carboxyl labeled acetic acid the heavy carbon was not evenly distributed between the two carboxyl groups (183). The C^{13} content of the carboxyl adjacent to the amino-group accounted for only one-fourth of the total, whereas it should have contained half of the total if the α-ketoglutarate arose from a symmetrical precursor. Thus, in the intact animal also, the α-ketoglutarate formed in the process of acetate oxidation is derived from an intermediate in which the orientation of the labeled carbon is preserved. The much higher isotope content of glutamic than of aspartic acid isolated from this experiment suggests that only a small fraction of the isotope incorporated into the glutamic acid was contributed by CO_2 assimilation. The carbon chain of aspartic acid is believed to be furnished by oxalacetate. If isotope had entered exclusively in the form of CO_2 by the carboxylation of pyruvate to oxalacetate, then aspartate should contain not less but as much or more heavy carbon than the α-ketoglutaric acid which is subsequently formed from oxalacetate by way of a tricarboxylic acid.

The results obtained with labeled substances eliminate a symmetrical molecule as the initial condensation product, but do not indicate which tricarboxylic acid is involved. In addition to isocitrate and cis-aconitate, the two acids which according to present views are the most likely intermediates, oxalosuccinate has become a possible intermediate since Ochoa (168) has demonstrated reversibility of the isocitrate-oxalosuccinate conversion. Citric acid has a symmetrical carbon chain but the possibility still exists that a citric acid which is substituted at one of the two primary carboxyl groups lies on the main path of the cycle. The suggestion of Wieland and Rosenthal (176) that the citric acid formed in kidney from acetoacetate might have arisen secondarily from a procitric acid containing eight carbon atoms has been mentioned. Lynen (188) finds the formation of citrate from acetate and oxalacetate by yeast to be promoted by the simultaneous oxidation of succinaldehyde and believes that the function of this reaction is to convert either one of the components into a more reactive derivative. If the condensation takes place with an acetyl compound CH_3 COR, a substituted unsymmetrical citrate molecule may result, which could be converted to cis-aconitate without elimination of the substituent R. Lynen has pointed out that the data of Sonderhoff and Thomas (127) on the formation of labeled citrate and succinate in yeast, also rule out a symmetrical citric acid. Succinate formed from trideuterioacetate by way of a symmetrical tricarboxylic acid could not have contained more than one atom of deuterium, but was actually found to contain two atoms. The suggestion that an unsymmetrically substituted citric acid derivative is initially formed would also account for the unequal distribution of isotopic carbon in α-ketoglutarate formed from labeled acetate or acetoacetate. The question concerning the identity of the initial reaction product might be clarified materially if it were possible to establish whether citrate formation occurs only in systems which contain the enzyme aconitase. According to Krebs (162) this enzyme is absent from yeast. In this case the citric acid arising in the course of acetate oxidation by this organism could not have been formed secondarily from isocitrate or cis-aconitate but must have been the primary condensation product.

A discussion of the rôle of the citric acid cycle in carbohydrate metabolism is beyond the scope of this review, but it may be pointed out that no evidence exists to support the contention that the tricarboxylic acid formed in the oxidative metabolism of pyruvate is identical with that arising from fatty acid oxidation. The finding of Martius (189) that oxalocitramalate, the condensation product of pyruvate and oxalacetate, is not attacked by kidney tissue is hardly sufficient evidence to rule out the occurrence of intermediate C_7 tricarboxylic acids. As the experience with yeast has shown (190, 157), the lack of metabolic activity of a substance added to a biological system may be apparent only and

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may be c... d by inability of the substance to penetrate cell walls. This is particularly true for polycarboxylic acids. Furthermore, there are other C_7 acids which could conceivably arise from pyruvate and oxaloacetate and which have not been tested biologically (formylcitric, oxalocitraconic acids).

The conclusion that the tricarboxylic acid cycle provides an important mechanism for the complete oxidation of fatty acids in the intact animal is based on evidence of indirect nature. The appearance of isotopic carbon in the glycogen of animals fed labeled fatty acids and in the dicarboxylic amino acids after feeding of acetate is not by itself indicative of the mechanism responsible, but is most reasonably ascribed to pathways which lead to the intermediary formation of tricarboxylic acids. It is not possible at present to determine what portion of the fatty acids is oxidized in the intact animal by this route. From the high isotope level in glycogen in the experiments of Buchanan et al. (120) and of Lorber et al. (121) it is clear however that a significant fraction of acetate and butyrate respectively was metabolized by way of the tricarboxylic acid cycle. In these experiments with starved animals, ordinary glucose was given simultaneously with the labeled fatty acids in order to insure glycogen deposition. The labeled glycogen arising from the labeled fatty acids was therefore diluted by glycogen formed directly from dietary glucose.

The finding of Wood et al. (182) that the same isotope distribution in glucose obtains with butyrate labeled at carbon atoms 1 and 3 as with carboxyl labeled acetate demonstrates the breakdown of butyrate to two acetyl groups and furnishes additional evidence for the view that fatty acids are degraded by successive removal of two carbon units. These data are not in agreement with the suggestion of Blixenkrone-Moeller (191) that butyrate might be oxidized to succinate directly by ω oxidation.

The results obtained during the past few years have not only provided the basis for a reasonable scheme of the total oxidation of fatty acids but have also clarified the controversial relationship of fat and carbohydrate metabolism. From the findings discussed above it may be concluded that fatty acids are convertible to carbohydrate in a restricted sense. The split products of fatty acid oxidation contribute to carbohydrate synthesis but the fatty acids by themselves are incapable of causing an increased formation of glucose or glycogen because the mechanism by which the fatty acid carbons find their way into glucose requires the participation of carbohydrate itself. This explains why in the starved or diabetic animal fatty acids and acetate do not show a glycogenic effect.

REFERENCES

(1) Klein, J. R. and J. S. Harris. J. Biol. Chem. **124**: 613, 1938.
(2) Lipmann, F. J. Biol. Chem. **160**: 173, 1945.
(3) Lole, A. Biochem. Ztschr. **47**: 118, 1912.
(4) Monguio, J. Klin. Wchnschr. **31**: 1116, 1934.
(5) MacKay, E. M., R. H. Barnes, H. O. Carne and A. N. Wick. J. Biol. Chem. **135**: 157, 1940.
(6) Bray, H. G., F. C. Neale and W. V. Thorpe. Biochem. J. **40**: 406, 1946.
(7) Litchfield, J. T. J. Pharmacol. **67**: 212, 1939.

(8) Knoop, F. Ztschr. physiol. Chem. **67**: 489, 1910.
(9) Marshall, E. K., Jr., K. Emerson, Jr. and W. C. Cutting. J. A. M. A. **110**: 252, 1938.
(10) Stekol, J. A. Ann. Rev. Biochem. **10**: 265, 1941.
(11) Handler, P. and W. A. Perlzweig. Ann. Rev. Biochem. **14**: 617, 1945.
(12) Green, D. E., D. H. Moore, V. Nocito and S. Ratner. J. Biol. Chem. **156**: 383, 1944.
(13) Cohn, R. Ztschr. physiol. Chem. **17**: 284, 1893.
(14) Neubauer, O. and O. Warburg. Ztschr. physiol. Chem. **70**: 1, 1910.
(15) Knoop, F. and J. G. Blanco. Ztschr. physiol. Chem. **146**: 267, 1925.
(16) du Vigneaud, V. and C. E. Meyer. J. Biol. Chem. **93**: 295, 1932.
(17) du Vigneaud, V. and O. J. Irish. J. Biol. Chem. **122**: 349, 1937.
(18) Erlenmeyer, E. and J. Kunlin. Annalen **307**: 146, 1899.
(19) de Jong, A. W. K. Rec. trav. chim. Pays-bas **19**: 259, 1900.
(20) du Vigneaud, V., M. Cohn, G. B. Brown, O. J. Irish, R. Schoenheimer and D. Rittenberg. J. Biol. Chem. **131**: 273, 1939.
(21) Bernhard, K. Ztschr. physiol. Chem. **267**: 91, 1940.
(22) du Vigneaud, V., J. L. Wood and O. J. Irish. J. Biol. Chem. **129**: 171, 1939.
(23) du Vigneaud, V., J. L. Wood and F. Binkley. J. Biol. Chem. **138**: 369, 1941.
(24) Doisy, E. A., Jr. and W. W. Westerfeld. J. Biol. Chem. **149**: 229, 1943.
(25) Martin, G. J. and E. H. Rennebaum. J. Biol. Chem. **151**: 417, 1943.
(26) James, G. V. Biochem. J. **33**: 1688, 1939.
(27) Bernhard, K. Ztschr. physiol. Chem. **267**: 99, 1940.
(28) Bernhard, K. Ztschr. physiol. Chem. **273**: 31, 1941.
(29) Bloch, K. and D. Rittenberg. J. Biol. Chem. **155**: 243, 1944.
(30) Bloch, K. and D. Rittenberg. J. Biol. Chem. **159**: 45, 1945.
(31) Bloch, K. and E. Borek. J. Biol. Chem. **164**: 483, 1946.
(32) Krebs, H. A. Ann. Rev. Biochem. **5**: 254, 1936.
(33) Annau, E. Ztschr. physiol. Chem. **224**: 141, 1934.
(34) Edson, N. L. Biochem. J. **29**: 2082, 1935.
(35) Bloch, K. and D. Rittenberg. Fed. Proc. **5**: 122, 1946.
(36) Bergmann, M., V. du Vigneaud and L. Zervas. Ber. Chem. Ges. **62**: 1909, 1929.
(37) Bergmann, M. and L. Zervas. Ztschr. physiol. Chem. **175**: 145, 1928.
(38) Borsook, H. and H. M. Huffman. In C. L. A. Schmidt. The chemistry of the amino acids and proteins. p. 865, 2nd ed., Springfield, 1944.
(39) Zief, M. and J. T. Edsall. J. Am. Chem. Soc. **59**: 2247, 1937.
(40) Neuberger, A. and F. Sanger. Biochem. J. **37**: 515, 1943.
(41) Berg, C. P., W. C. Rose and C. S. Marvel. J. Biol. Chem. **85**: 207, 1932.
(42) du Vigneaud, V., R. R. Sealock and C. Van Etten. J. Biol. Chem. **98**: 565, 1932.
(43) Carter, H. E., P. Handler, F. Binkley, H. Fishbeck, W. Risser and J. Weisinger. Proc. Soc. Biol. Chem. **32**: xx, 1938.
(44) Neuberger, A. and T. A. Webster. Biochem. J. **40**: 576, 1946.
(45) Abbot, L. D., Jr. J. Biol. Chem. **145**: 241, 1942.
(46) Jackson, R. W. and J. P. Chandler. Ann. Rev. Biochem. **8**: 261, 1939.
(47) Weismann, N. and R. Schoenheimer. J. Biol. Chem. **140**: 779, 1941.
(48) Berg, C. P. J. Nutrition **12**: 671, 1936.
(49) Berg, C. P. J. Biol. Chem. **104**: 373, 1934.
(50) du Vigneaud, V., H. S. Loring and H. A. Craft. J. Biol. Chem. **107**: 519, 1934.
(51) Berg, C. P. and M. Potgieter. J. Biol. Chem. **94**: 661, 1932.
(52) Rose, W. C. Science **86**: 298, 1937.
(53) Knoop, F. Beitr. chem. Physiol. u. Path. **6**: 150, 1905.
(54) Dakin, H. D. J. Biol. Chem. **6**: 221, 1909.
(55) Thunberg, T. Skand. Arch. Physiol. **40**: 1, 1920.
(56) McClendon, J. F. J. Biol. Chem. **154**: 357, 1944.
(57) Cook, R. P. and K. Harrison. Biochem. J. **30**: 1640, 1936.

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(58) STADIE, W. C., J. A. ZAPP AND F. D. W. LUKENS. J. Biol. Chem. 137: 75, 1941.
(59) LEHNINGER, A. L. J. Biol. Chem. 143: 147, 1942.
(60) STADIE, W. C. Physiol. Rev. 25: 395, 1945.
(61) RITTENBERG, D. AND G. L. FOSTER. J. Biol. Chem. 133: 737, 1940.
(62) LORBER, V., N. LIPSON, H. G. WOOD AND J. BARCROFT. J. Physiol. 145: 557, 1946.
(63) RINGER, H. F., W. GOLDFARB, N. RAKERTEN, L. H. NAHUM AND D. DU BOIS. Am. J. Physiol. 110: 332, 1934.
(64) TAUBER, P. R. AND P. D. MCMASTER. J. exper. Med. 49: 765, 1929.
(65) STADIE, W. C. J. Clin. Investigation 19: 843, 1940.
(66) LEHNINGER, A. L. J. Biol. Chem. 165: 131, 1946.
(67) BERNFELD, K. AND F. BULLET. Helv. chim. acta 26: 1185, 1943.
(68) LEBOIR, L. F. AND J. M. MUÑOZ. Biochem. J. 33: 734, 1939.
(69) BUTTS, J. S., C. H. CUTLER, L. F. HALLMANN AND H. J. DEUEL, JR. J. Biol. Chem. 109: 597, 1935.
(70) DEUEL, H. J., JR., L. F. HALLMANN AND J. S. BUTTS. J. Biol. Chem. 116: 621, 1937.
(71) QUASTEL, J. H. AND A. H. M. WHEATLEY. Biochem. J. 27: 1753, 1933.
(72) JOWETT, M. AND J. H. QUASTEL. Biochem. J. 29: 2159, 1935.
(73) COHEN, P. P. J. Biol. Chem. 119: 333, 1937.
(74) LEHNINGER, A. L. J. Biol. Chem. 157: 363, 1945.
(75) SCHOENHEIMER, R. AND D. RITTENBERG. J. Biol. Chem. 120: 155, 1937.
(76) WEINHOUSE, S., G. MEDES AND N. F. FLOYD. J. Biol. Chem. 155: 143, 1944.
(77) MACKAY, E. M., R. H. BARNES, H. O. CARNE AND A. N. WICK. J. Biol. Chem. 136: 503, 1940.
(78) BLOCH, K. J. Biol. Chem. 155: 255, 1944.
(79) BUTTS, J. S., K. BLUNDEN AND M. S. DUNN. J. Biol. Chem. 120: 284, 1937.
(80) ROSE, W. C., T. E. JOHNSON AND W. T. HAINES. J. Biol. Chem. 145: 679, 1942.
(81) FRIEDMANN, F. Biochem. Ztschr. 55: 436, 1913.
(82) SCHNEIDER, M. E., R. H. BARNES, A. HEMINGWAY AND A. O. NIER. J. Biol. Chem. 142: 47, 1942.
(83) MEDES, G., S. WEINHOUSE AND N. F. FLOYD. J. Biol. Chem. 157: 751, 1945.
(84) WEINHOUSE, S., G. MEDES AND N. F. FLOYD. J. Biol. Chem. 158: 411, 1945.
(85) MEDES, G., S. WEINHOUSE AND N. F. FLOYD. J. Biol. Chem. 157: 35, 1944.
(86) MEDES, G., N. F. FLOYD AND S. WEINHOUSE. J. Biol. Chem. 162: 1, 1945.
(87) MORRIHOUSE, M. G. J. Biol. Chem. 129: 769, 1939.
(88) MORRIHOUSE, M. G. AND H. J. DEUEL, JR. Proc. Soc. Exper. Biol. 45: 96, 1941.
(89) LEHNINGER, A. L. J. Biol. Chem. 161: 413, 1945.
(90) KREBS, H. A. AND W. A. JOHNSON. Biochem. J. 31: 645, 1937.
(91) WELLS-MARTHURBE, H. Biochem. J. 31: 2292, 1937.
(92) LONG, C. Biochem. J. 32: 1711, 1938.
(93) LIPMANN, F. Skand. Arch. Physiol. 76: 255, 1937.
(94) LIPMANN, F. Cold Spring Harbor Symposia Quant. Biol. 7: 248, 1939.
(95) BARRON, E. S. G., G. R. BARTLETT AND G. KALNITSKY. Fed. Proc. 5: 120, 1946.
(96) EVANS, E. A., JR. Biochem. J. 34: 829, 1940.
(97) RITTENBERG, D., E. BOREK AND K. BLOCH. Fed. Proc. 5: 151, 1946.
(98) SOMLE, J. C., J. M. BUCHANAN AND A. M. DELLUVA. J. Biol. Chem. 166: 395, 1946.
(99) SHAREMAN, M. AND C. H. WERKMAN. J. Biol. Chem. 138: 35, 1940.
(100) GEIER, D. E., W. W. WESTERFIELD, B. VENNESLAND AND W. E. KNOX. J. Biol. Chem. 140: 653, 1941.
(101) GREENBERG, L. J. Pharmacol 77: 194, 1943.
(102) WESTERFIELD, W. W. AND R. L. BERG. J. Biol. Chem. 148: 523, 1943.
(103) DIETZER, M. Annalen 530: 44, 1938.
(104) SLADE, H. D. AND C. H. WERKMAN. J. Bact. 41: 675, 1940.
(105) CAMPBELL, J. J. R. AND I. C. GUNSALUS. J. Bact. 48: 71, 1944.

(106) GUNSALUS, I. C. AND J. J. R. CAMPBELL. J. Bact. 48: 455, 1944.
(107) LIPTON, M. A. AND E. S. G. BARRON. J. Biol. Chem. 166: 367, 1946.
(108) SLADE, H. D. AND C. H. WERKMANN. Arch. Biochem. 2: 97, 1943.
(109) ROBINSON, R. J. Soc. Chem. Ind. 53: 1062, 1934.
(110) FRASER, M. T. AND J. A. GARDNER. Proc. Roy. Soc. B. 82: 55, 1910.
(111) WINDAUS, A. Cited in H. LETTRÉ AND H. H. INHOFFEN. Ueber Sterine, Gallensäuren und Verwandte Naturstoffe. Stuttgart 1936, p. 103.
(112) RITTENBERG, D. AND R. SCHOENHEIMER. J. Biol. Chem. 121: 235, 1937.
(113) SHEMIN, D. AND D. RITTENBERG. J. Biol. Chem. 159: 567, 1945.
(114) SHEMIN, D. AND D. RITTENBERG. J. Biol. Chem. 166: 621, 1946.
(115) WOOD, H. G., N. LIPSON AND V. LORBER. J. Biol. Chem. 159: 475, 1945.
(116) STETTEN, D., JR. AND M. R. STETTEN. J. Biol. Chem. 165: 147, 1946.
(117) RITTENBERG, D. AND K. BLOCH. J. Biol. Chem. 154: 311, 1944; 160: 417, 1945.
(118) BLOCH, K. AND D. RITTENBERG. J. Biol. Chem. 145: 625, 1942.
(119) DEUEL, H. J., JR. AND A. T. MILHORAT. J. Biol. Chem. 78: 299, 1928.
(120) BUCHANAN, J. M., A. B. HASTINGS AND F. B. NESBETT. J. Biol. Chem. 150: 413, 1943.
(121) LORBER, V., N. LIPSON AND H. G. WOOD. J. Biol. Chem. 161: 411, 1945.
(122) BUCHANAN, J. M. AND A. B. HASTINGS. Physiol. Rev. 26: 120, 1946.
(123) WOOD, H. G. Physiol. Rev. 26: 198, 1946.
(124) EMBDEN, G. AND F. OPPENHEIMER. Biochem. Ztschr. 45: 186, 1912.
(125) LEHNINGER, A. L. J. Biol. Chem. 164: 291, 1946.
(126) MACLEAN, I. S. AND D. HOFFERT. Biochem. J. 20: 343, 1926.
(127) SONDERHOFF, R. AND H. THOMAS. Annalen 530: 195, 1937.
(128) WOOD, H. G., R. W. BROWN AND C. H. WEREMANN. Arch. Biochem. 6: 243, 1945.
(129) BARKER, H. A., M. D. KAMEN AND B. T. BORNSTEIN. Proc. Natl. Ac. Sci. 31: 373, 1945.
(130) STETTEN, D., JR. AND R. SCHOENHEIMER. J. Biol. Chem. 133: 329, 1940.
(131) BLOCH, K., E. BOREK AND D. RITTENBERG. J. Biol. Chem. 162: 441, 1946.
(132) STETTEN, D., JR. AND G. E. BOXER. J. Biol. Chem. 156: 271, 1944.
(133) BLOCH, K. AND D. RITTENBERG. J. Biol. Chem. 143: 297, 1942.
(134) BLOCH, K., B. N. BERG AND D. RITTENBERG. J. Biol. Chem. 149: 51, 1943.
(135) BLOCH, K. J. Biol. Chem. 157: 661, 1945.
(136) BLOCH, K. AND D. RITTENBERG. Unpublished.
(137) SHEMIN, D. AND D. RITTENBERG. J. Biol. Chem. 166: 627, 1946.
(138) FISCHER, H. AND E. FINK. Ztschr. physiol. Chem. 280: 123, 1944.
(139) KNOOP, L. Ber. Chem. Ges. 17: 1638, 1884.
(140) BARKER, H. A. AND J. V. BECK. J. Biol. Chem. 141: 3, 1941.
(141) ALMQUIST, H. J., E. L. R. STOKSTAD, E. MECCHI AND P. D. V. MANNING. J. Biol. Chem. 134: 213, 1940.
(142) GUIRARD, B. M., E. E. SNELL AND R. J. WILLIAMS. Arch. Biochem. 9: 361, 1946.
(143) SCHOENHEIMER, R. The dynamic state of body constituents. Harvard University Press, Cambridge, 1942.
(144) KHARASCH, M. S. AND M. T. GLADSTONE. J. Am. Chem. Soc. 65: 15, 1943.
(145) UTTER, M. F., F. LIPMANN AND C. H. WERKMAN. J. Biol. Chem. 158: 521, 1945.
(146) DU VIGNEAUD, V. The Harvey Lectures, Series 38: p. 39, 1942.
(147) BORSOOK, H. AND J. W. DUBNOFF. Science 91: 551 1940.
(148) BLOCH, K. AND R. SCHOENHEIMER. J. Biol. Chem. 134: 785 1940.
(149) SOLOMON, A. K., B. VENNESLAND, F. W. KLEMPERER, J. M. BUCHANAN AND A. B. HASTINGS. J. Biol. Chem. 140: 171, 1941.
(150) ELLIOT, K. A. C., M. B. BENOY AND Z. BAKER. Biochem. J. 29: 1936, 1935.
(151) TOENIESSEN, E. AND E. BRINKMANN. Ztschr. physiol. Chem. 252: 169, 1938.
(152) KLEINZELLER, A. Biochem. J. 37: 674, 1944.
(153) WIELAND, H. AND R. SONDERHOFF. Annalen 499: 213, 1932.

(154) Lipmann, F. Advances in Enzymology, New York **1**: 99, 1941.
(155) Wieland, H., G. Jennen and W. Schwarze. Annalen **548**: 265, 1941.
(156) Jowett, M. and J. H. Quastel. Biochem. J. **29**: 2181, 1935.
(157) Lynen, F. Annalen **554**: 40, 1943.
(158) Breusch, F. L. Science **97**: 490, 1943.
(159) Weil-Malherbe, H. Nature **153**: 435, 1944.
(160) Krebs, H. A. and L. Eggleston. Nature **154**: 209, 1944.
(161) Werkmann, C. H. and H. G. Wood. Advances in Enzymology, New York **2**: 135, 1942.
(162) Krebs, H. A. Advances in Enzymology, New York **3**: 191, 1943.
(163) Evans, E. A., Jr. The Harvey Lecture, Series **39**: 273, 1944.
(164) Lipmann, F. Advances in Enzymology, New York **6**: 231, 1946.
(165) Lipmann, F. J. Biol. Chem. **155**: 55, 1944.
(166) Lipmann, F. and C. L. Tuttle. J. Biol. Chem. **161**: 415, 1945.
(167) Buchanan, J. M. and J. C. Sonne. J. Biol. Chem. **166**: 781, 1946.
(168) Ochoa, S. J. Biol. Chem. **159**: 243, 1945.
(169) Krebs, H. A. and W. A. Johnson. Biochem. J. **31**: 772, 1937.
(170) Lehninger, A. L. J. Biol. Chem. **148**: 393, 1943.
(171) Krebs, H. A. Nature **138**: 288, 1936.
(172) Weinhouse, S., G. Medes, N. Floyd and L. Noda. J. Biol. Chem. **161**: 745, 1945.
(173) Martius, C. Ztschr. Physiol. Chem. **257**: 29, 1938.
(174) Krebs, H. A. Biochem. J. **36**: ix, 1942.
(175) Weinhouse, S., G. Medes and N. Floyd. J. Biol. Chem. **166**: 691, 1946.
(176) Wieland, H. and C. Rosenthal. Annalen **554**: 211, 1943.
(177) Hunter, E. and L. F. Leloir. J. Biol. Chem. **159**: 255, 1945.
(178) Virtanen, A. J. Ann. Ac. Sc. Fenn. **33**: 3, 1930.
(179) Sonderhoff, R. and M. Deffner. Annalen **530**: 195, 1937.
(180) Buchanan, J. M., W. Sakami, S. Gurin and D. W. Wilson. J. Biol. Chem. **159**: 695, 1945.
(181) Breusch, F. L. Enzymologia **11**: 169, 1944.
(182) Wood, H. G., N. Lifson and V. Lorber. Unpublished results, cited in Physiol. Rev. **26**: 198, 1946.
(183) Rittenberg, D. and K. Bloch. J. Biol. Chem. **157**: 749, 1945.
(184) Buchanan, J., W. Sakami, S. Gurin and D. W. Wilson. Fed Proc. **5**: 126, 1946.
(185) Krebs, H. A. and W. A. Johnson. Enzymologia **4**: 148, 1937.
(186) Evans, E. A., Jr. and L. Slotin. J. Biol. Chem. **141**: 439, 1941.
(187) Wood, H. G., C. H. Werkmann, A. Hemingway and A. O. Nier. J. Biol. Chem. **142**: 31, 1942.
(188) Lynen, F. Annalen **552**: 272, 1942.
(189) Martius, C. Ztschr. physiol. Chem. **274**: 96, 1943.
(190) Hahn, L., G. Hevesy and E. Lundsgaard. Biochem. J. **31**: 1705, 1937.
(191) Blixenkrone-Moeller, N. Ztschr. physiol. Chem. **252**: 137, 1938.

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ON THE UTILIZATION OF ACETIC ACID FOR CHOLESTEROL FORMATION*

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The information at present available as to the metabolism of cholesterol and its relationship to the other biologically important sterols is meager. Balance experiments (1) have demonstrated that cholesterol can be synthesized and destroyed in the animal but have given no convincing evidence as to the nature of specific precursors of cholesterol.

In general, balance experiments fail to differentiate between two alternatives, direct utilization of the dietary component or stimulation of a specific process of metabolism. It cannot, for example, be decided whether the increase of cholesterol observed in some experiments after the feeding of fatty substances is the result of conversion of these substances to cholesterol or whether it merely reflects an increased metabolic activity of the organism. An experiment (2) on the rate of formation of cholesterol, carried out with the aid of deuterium, indicated that about half of all the hydrogen atoms of the newly formed cholesterol was derived from the hydrogen of water; the other half must have originated from the carbon-bound hydrogen atoms of some dietary constituent. This evidence eliminated large molecules as immediate precursors, and suggested that "cholesterol . . . is formed by the coupling of smaller molecules, possibly those which have been postulated to be intermediates in the fat and carbohydrate metabolism" (2).

The recent observation (3) that the ingestion of deuterio acetic acid, CD_3COOH, leads to the formation of deuterio cholesterol supports this view. On the other hand, deuterio cholesterol is not formed after α,β-dideuterio propionic acid and deuterio succinic acid are fed. α,β-Dideuterio butyric acid and β,γ-dideuterio butyric acid were only slightly effective. The deuterium concentrations in the total cholesterol of the experimental animals after the feeding of deuterio acetate for 8 days are given in Table I. Positive evidence for the utilization of acetate for cholesterol formation was found for mice, growing rats, and adult rats. In each case the concentration of deuterium in the cholesterol was over 3 times that in the body water. The figures in the seventh column give the proportion

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of all the hydrogen in the cholesterol which was derived from the dietary acetate during the experimental period. These values must of necessity be small, if only for the reason that the rate of synthesis of cholesterol is slow.

In considering possible steps in the transformation of acetate to sterol, a mechanism must be postulated in which the acetate molecules are reductively coupled to form larger units. Intermediates containing only labile hydrogen, *i.e.* readily exchangeable hydrogen, can be ruled out. For example, a pathway via oxalacetic acid cannot be considered, as it would result in the elimination of deuterium by enolization. We have tested some compounds into which acetic acid is known to be converted *in vivo* in order to determine whether any of these reactions is involved in sterol synthesis.

TABLE I

Feeding of Deuterio Acetic Acid

Experiment	Animals used	Deuterio acetic acid fed per day per 100 gm. body weight	Deuterium in			Hydrogen in cholesterol derived from acetate	Deuterium in total fatty acids
			Sodium acetate	Body water	Isolated cholesterol		
		mg.	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*	*per cent*	*atom per cent excess*
A	Adult mice	372	9.9	0.04	0.13	1.3	0.02
B	Young rats	82	68.0	0.08	0.27	0.4	0.04
C	Adult "	97	27.6	0.05	0.21*	0.8	

* In this experiment the cholesterol was converted to cholesteryl chloride, which was analyzed.

Yeast dehydrogenases rapidly convert acetate to succinate (4). As the results obtained on feeding deuterio succinate were negative, the pathway from acetate to cholesterol does not go through succinic acid.

A conceivable explanation for the failure of succinate to generate deuterio cholesterol might be that the succinic-fumaric-malic equilibrium causes a rapid loss of deuterium to the body fluids and therefore renders detection of cholesterol formation impossible. However, this cannot be the reason. Assuming that acetate were converted to succinate and that the latter lost deuterium by exchange, then deuterio acetate should not be more effective in forming deuterio cholesterol than deuterio succinate. Succinate does not represent an intermediate step.

Little is known of acetate metabolism in normal animals. It has apparently been believed, but not proved, that acetic acid is directly burned to carbon dioxide and water at a rapid rate. Under certain conditions, such as in fasting animals (5) or in liver slices (6), formation of aceto-

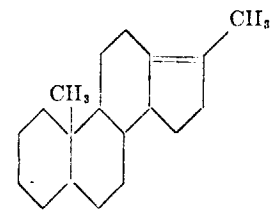
acetic acid and β-hydroxybutyric acid from acetic acid has been demonstrated and ascribed to condensation of acetic acid with either pyruvic acid (7) or another molecule of acetic acid (8).

Mowbouse (9) has isolated deuterio β-hydroxybutyric acid from the urine of fasting rats which had been given β,γ-dideuterio butyric acid, but found normal β-hydroxybutyric acid after α,β-dideuterio butyric acid was fed. This demonstrates that, while the α- and β-hydrogen atoms are rapidly replaced, the γ-hydrogen atoms are relatively stable. β,γ-Dideuterio butyric acid can be expected to give rise to deuterio acetoacetic acid in normal animals also. When its sodium salt was fed to rats, the isotopic content of the cholesterol was only slightly higher than that of the body fluids in contrast to a comparable feeding of deuterio acetate, when the cholesterol formed contained 3 to 4 times more deuterium than the surrounding body water. In view of the known relationship of butyric, acetoacetic and β-hydroxybutyric acids these compounds may be ruled out as intermediates in sterol formation from acetic acid.

In another experiment our animals received a corresponding amount of α,β-dideuterio butyric acid. This compound was expected to exchange its deuterium by enolization after being oxidized to acetoacetic acid, and the cholesterol isolated should not contain significant concentrations of deuterium. The concentration of deuterium in the isolated cholesterol was slightly higher than that of the body fluids.

Propionic acid was tested as a possible cholesterol precursor, because of its chemical relationship to pyruvic acid. Sodium α,β-dideuterio propionate was fed to rats as a source of deuterio pyruvic acid, but failed to produce a cholesterol with a significant deuterium content. If this failure were due to removal of deuterium by enolization, the same loss of isotope should occur with acetate if it were converted to cholesterol via pyruvate. Our experimental results indicate that propionic acid and presumably pyruvic acid are not intermediates in the synthesis of cholesterol from acetate.

We have attempted, by chemical degradation of the deuterio cholesterol, to obtain some more detailed information on the mechanism of sterol synthesis. Unfortunately the yields of cholesterol fragments which could be obtained by oxidative breakdown of the available deuterio cholesterol samples would be too small to permit deuterium analysis. The only reaction found to be suitable for our purpose was the thermal degradation of cholesteryl chloride described by Mauthner (10). When this compound is heated to about 300° in a nitogen atmosphere, hydrochloric acid is eliminated; at about 400° fission occurs between carbon atoms 17 and 20, yielding isooctane, isooctene, and a high boiling oil to which the structure shown in the accompanying formula has been assigned by Bergmann and Bergmann (11).



By converting the deuterio cholesterol isolated from our animals into cholesteryl chloride and subjecting the latter to the thermal degradation, we have obtained two fractions, representing the side chain and the sterol nucleus respectively. The concentrations of deuterium in the isooctane-isooctene mixture and in the hydrocarbon $C_{19}H_{30}$, which may be regarded as the primary degradation products, are in good agreement with that in the cholesteryl chloride. The occurrence of side reactions leading to a loss of fractions with high deuterium concentrations is therefore unlikely. Small errors in the calculation may arise from two sources. The hydrochloric acid split off in the initial phase of the degradation was not analyzed. However, unless the deuterium concentration at carbon atom 2 or 4 of the sterol nucleus was very much higher than that at other carbon atoms the analytical figure for the nucleus could not have been influenced profoundly. Secondly, the high boiling hydrocarbon $C_{19}H_{30}$ contains only one double bond instead of the two or three expected. The drastic treatment of the cholesteryl chloride evidently involves hydrogen shifts, which, however, should not greatly change the isotopic concentration of the fragments unless the deuterium concentration at any particular carbon is of a different order of magnitude from that at others.

From the analytical values obtained for the two breakdown products, it becomes evident that acetate had been utilized for the biosynthesis of the side chain as well as the steroid nucleus. Indeed the deuterium concentration found in the side chain exceeds that of the nucleus by approximately 50 per cent. The difference may arise from the fact that the methyl groups of the cholesterol at positions 18, 19, 21, 26, and 27 may originate directly from the acetic acid with the retention of all 3 hydrogen atoms, while the other carbon atoms probably retain only 1 or 2 of the original hydrogen atoms. Since the methyl groups account for 50 per cent of all the hydrogen in the side chain, and for only 20 per cent of the total hydrogen in the nucleus, more of the deuterium could be expected to be incorporated into the side chain.[1]

[1] The foregoing considerations ignore the possibility that intramolecular hydrogen shift had occurred between the side chain and the nucleus during the pyrolysis. This, however, cannot be excluded at present. As the deuterium content of the isooctane is found to be higher than that of the ring hydrocarbon, the isotope must

EXPERIMENTAL

Preparation of Deuterio Compounds

Deuterio Acetic Acid—Deuterio malonic acid was prepared by exchange with heavy water and decarboxylated to deuterio acetic acid (12). For feeding experiments the sodium salt was used.

α,β-Dideuterio propionic acid was prepared from methyl acrylate as previously described (13). The sodium salt of the acid contained 34.4 atom per cent excess deuterium.

α,β-Dideuterio butyric acid was prepared by hydrogenating ethyl crotonate with deuterium gas. The sodium butyrate contained 23.1 atom per cent excess deuterium.

β,γ-Dideuterio butyric acid was similarly prepared from vinylacetic acid. The sodium salt of the β,γ-dideuterio butyric acid contained 16.0 atom per cent excess deuterium. As the β,γ double bond in vinylacetic acid is known to shift easily towards the α,β position (14), it was felt necessary to prove that the isotope was actually located at the β- and γ-carbon atoms. To [illegible] gm. of the sodium butyrate (3.57 mM) in water, 10.34 gm. (117.5 mM) of ordinary butyric acid and an excess of sulfuric acid were added. The butyric acid mixture was extracted from the aqueous solution by ether and the ether dried and distilled off. The butyric acid thus obtained was brominated according to the method of Fischer (15). The α-bromobutyric acid obtained distilled at 120–122°, 19 mm. It contained 0.49 atom per cent excess deuterium or 16.6 per cent when calculated for the undiluted acid. This compares with a value of 16.0 per cent for the deuterium analysis of the sodium β,γ-dideuterio butyrate. As the sodium butyrate and the α-bromobutyric acid both contain 7 hydrogen atoms, the agreement between the two values demonstrates that no deuterium had been lost as the result of the bromination; *i.e.*, no shift of double bonds from the β,γ to the α,β positions had occurred during the catalytic hydrogenation of vinylacetic acid.

Isolation of Cholesterol—Cholesterol was obtained in the usual manner from the unsaponifiable fractions of the total pooled carcasses. The samples were converted into the dibromides, debrominated (16), and recrystallized. The melting points of the purified cholesterol samples ranged from 147–149°.

Cholesterol Degradation—1.3 gm. of crude sterol obtained from Experiment C, Table I, were converted into cholesteryl chloride by dissolving in

have been present in the cholesterol side chain prior to the degradation. The same, however, does not hold necessarily for the sterol nucleus. The amounts of deuterio cholesterol available at present do not permit as yet the carrying out of reactions from which the deuterium content at individual carbon atoms could be determined.

chloroform and adding 0.6 gm. of freshly distilled thionyl chloride. The mixture was refluxed for 1 hour, and the solvent and excess reagent were distilled off. The dark brown residue failed to crystallize. Purification was achieved by passing a petroleum ether solution of the crude product through a column of activated alumina. The colorless petroleum ether filtrate was evaporated and the residue crystallized from a small volume of acetone. There was obtained 0.75 gm. of cholesteryl chloride, m.p. 94–95°, containing 0.21 atom per cent excess deuterium.

$C_{27}H_{45}Cl$. Calculated, C 80.1, H 11.1; found, C 79.7, H 11.4

For the thermal degradation, 0.65 gm. of the deuterio cholesteryl chloride, diluted with an equal amount of non-isotopic cholesteryl chloride, was slowly heated to 390° in an atmosphere of nitrogen. Hydrochloric acid was evolved. The temperature was kept constant until the HCl evolution ceased (4 hours). The bath temperature was then slowly raised to about 400°. At this temperature a volatile product distilled over very slowly and was collected in a trap cooled by dry ice. About 250 mg. of a mobile distillate were obtained; it was redistilled into a second trap. The liquid was presumed to be a mixture of isooctane and isooctene. Its boiling range was 115–120°; *i.e.*, roughly the same range as that observed by Bergmann and Bergmann (11). For deuterium analysis the liquid was volatilized directly into the combustion furnace by a slow oxygen stream. The resulting water contained 0.128 atom per cent excess deuterium. Since the deuterio cholesteryl chloride had been diluted 1:1, the actual value for the isooctane-isooctene mixture is 0.256 atom per cent excess deuterium.

The residue which remained after the pyrolysis was heated over a free flame, and a viscous yellow oil distilled over between 380–400°. An attempt to obtain a crystalline hydrocarbon from this fraction was unsuccessful. 0.50 gm. of this high boiling oil was dissolved in petroleum ether and the solution passed through a column of activated alumina. Only 10 per cent of the fraction was adsorbed. The combined filtrate and petroleum ether washings were free of pigments. The colorless oil remaining after removal of the solvent had the following composition.

$C_{19}H_{30}$. Calculated, C 88.4, H 11.6; found, C 88.3, H 11.7
$[\alpha]_D^{25} = +30.7°$ (2% in benzene)

Bergmann and Bergmann reported C 87.9, H 11.9, $[\alpha]_D = +31.4°$. The hydrocarbon contained 0.089 atom per cent excess deuterium, or 0.178 per cent calculated for the undiluted starting material.

On the basis of data obtained by Bergmann and Bergmann, we assume that the composition of the volatile hydrocarbon corresponds closely to that of isooctane, C_8H_{18}. The average deuterium content of the hydrogen

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in a compound composed of the fragments C_8H_{15} and $C_{19}H_{30}$, as calculated from their isotope content, would be $(18 \times 0.26 + 30 \times 0.18)/48 = 0.21$ per cent. This compares well with the value 0.21 per cent found for the deuterium content of the hydrogen in the cholesteryl chloride which had been used as a starting material.

The catalytic hydrogenation of the hydrocarbon $C_{19}H_{30}$ with platinum in acetic acid consumed only one-third of the amount of hydrogen required for one double bond. The resistance to hydrogenation is in agreement with the view of Bergmann and Bergmann that the double bond in the hydrocarbon $C_{19}H_{30}$ is located at quaternary carbon atoms.

Animal Experiments

Feeding of Deuterio Acetic Acid. Experiment A—Seven adult mice received the following diet (Stock Diet I): 50 per cent casein, 20 per cent Wesson oil, 14 per cent salt mixture (17), 16 per cent yeast. The high salt content of the diet resulted in a large water consumption and urine excretion, thereby favoring the "washing out" of heavy water from the body fluids. In addition to the stock diet, each mouse received 110 mg. of sodium deuterio acetate (9.9 atom per cent excess deuterium) per day for 8 days. At the end of the experimental period, each mouse had gained an average of 4 gm. The animals were killed, body water was distilled from the tissues, and total fatty acids and cholesterol were isolated from the animal carcasses after removal of the gastrointestinal tracts.

Experiment B—Two growing rats, weighing 102 and 110 gm. respectively, were given the carbohydrate-free Stock Diet I and in addition 137 mg. of sodium deuterio acetate (68 atom per cent excess deuterium) per animal per day over a period of 8 days. Each animal gained about 30 gm. during the feeding period.

Experiment C—Four adult rats having a combined weight of 1113 gm. received the following Stock Diet II: 86 per cent casein, 3 per cent Wesson oil, 5 per cent yeast, 5 per cent salt mixture (17), 1 per cent cod liver oil. During the 8 day feeding period, each animal received 400 mg. of sodium deuterio acetate (27.6 atom per cent excess deuterium) per day. At the end of the experimental period, the combined weight of the animals was 1196 gm. A total of 1.4 gm. of crude cholesterol was obtained from the animal carcasses.

The results of the deuterium analysis of body water, fatty acids, and cholesterol are given in Table I. The data suggest that variation of dietary composition has had little effect on the formation of deuterio cholesterol. During the experimental period, the feces were collected from the animals of Experiment C, Table I. They were ground up with anhydrous sodium sulfate, extracted with ether and acetone, and the sterols precipitated from

the unsaponifiable fraction by digitonin. If we assume that the fecal sterols were derived from the body sterols and that the deuterium concentration in the body sterols increased linearly during the experiment, then the deuterium concentration of the total fecal sterol should be half of that of the body cholesterol at the end of the experiment. The fecal sterols recovered from the digitonides contained 0.110 atom per cent excess deuterium, as compared to 0.21 atom per cent excess in the body cholesterol.

Feeding of Deuterio Succinic Acid—Nine adult mice received the carbohydrate-free Stock Diet I and a daily addition per mouse of 110 mg. of deuterio succinic acid,[2] containing 26.9 atom per cent excess deuterium. During the experimental period the combined weight of the mice increased from 180 to 215 gm. Body water and cholesterol from the combined carcasses contained 0.13 and 0.07 atom per cent excess deuterium respectively.

TABLE II

Feeding of Deuterio Fatty Acids to Adult Rats

1.6 mM of the sodium salt of deuterio fatty acid were fed per day per 100 gm. of body weight in each case.

Compound fed (sodium salts)	Deuterium in compound fed	Deuterium in		
		Body water	Cholesterol	Fatty acids
	atom per cent excess	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*
Acetic acid	27.6	0.05	0.21	
α,β-Dideuterio propionic acid	34.4	0.05	0.02	0.01
" butyric acid	23.1	0.03	0.04	
β,γ-Dideuterio " "	16.0	0.05	0.07	0.02

Feeding of Sodium α,β-Dideuterio Propionate—Two rats having a combined weight of 297 gm. received the carbohydrate-free, low fat Stock Diet II. During the experimental period of 8 days, each animal received a daily addition of 231 mg. of sodium deuterio propionate (34.4 atom per cent excess deuterium). On the 3rd day of feeding, the rats developed a slight diarrhea, which disappeared when some bone meal was added to the diet. At the end of the experimental period, the two animals had a total weight of 340 gm. The analytical data are given in Table II.

Feeding of Sodium α,β-Dideuterio Butyrate—Two rats, having a total weight of 294 gm., were given Stock Diet II and in addition 265 mg. of sodium butyrate (23.1 atom per cent excess deuterium) per animal per day. No change of weight occurred during the 8 day feeding period. The deuterium analyses for body water and cholesterol are given in Table II.

[2] The deuterio succinic acid was kindly supplied by Dr. Abraham Mazur. It had been prepared from diethyl fumarate by catalytic hydrogenation with deuterium gas.

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Feeding of Sodium β,γ-Dideuterio Butyrate—Two rats received Stock Diet II and in addition 265 mg. of sodium butyrate (16.0 atom per cent excess deuterium) daily per rat over a period of 8 days. The combined weight of the animals increased from 291 to 329 gm. The analytical results are presented in Table II.

DISCUSSION

In all experiments the test substance was supplied with the diet for 8 days. During this period the animals were slowly synthesizing and destroying cholesterol. The rate of these reactions for the adult mouse is about 2.5 per cent per day (2); so that at the end of the 8 day period the total cholesterol, *i.e.* the cholesterol actually analyzed, should represent a mixture of about 1 part of newly formed and 4 parts of preexisting cholesterol. The rate of cholesterol synthesis in the rat has not been determined but may be assumed to be of similar magnitude. We know from the experiments on the deuterium content of cholesterol of mice whose body fluids contain D_2O that the maximum concentration of deuterium in their cholesterol is about half of that of the body fluids. A level of about one-tenth is reached in 8 days. Whenever in the present experiments the concentration of deuterium in the total cholesterol is appreciably over one-tenth that of the body fluids, there is presumptive evidence for a synthesis utilizing hydrogen from a deuterio dietary constituent and not from water alone.[3] The appearance of cholesterol with a concentration greater than half that of the body fluids is conclusive evidence for the utilization of the isotopic test substance in the synthesis of cholesterol, since no assumption need then be made as to the rate of this reaction. The results of the three acetic acid experiments satisfy both criteria for conversion of acetate to cholesterol. In each case the deuterium concentration is over 30 times that which could be expected to enter the cholesterol from the body fluids.

Our experimental results demonstrate clearly that the cyclopentanoperhydrophenanthrene structure can be synthesized from C_2 units. A condensation of numerous small molecules with the intermediate uptake and elimination of the elements of water, with or without reduction of double bonds, is therefore suggested. From our data the exact nature of the unit undergoing condensation cannot be stated. It seems unlikely to be the relatively inert acetic acid itself, but rather a closely related intermediate such as acetaldehyde,[4] into which acetic acid might readily be converted by the organism.

[3] The deuterium concentration of the body fluids was zero at the start of the experiment and at a maximum, given in Tables I and II, at the end. It may be assumed that the major part of the acetic acid was oxidized to water and carbon dioxide. From the amounts of deuterium administered and the fluid intake of the animals it can then be calculated that the deuterium concentration in the body fluids rose for about 3 days and thereafter remained relatively constant.

The available data are not sufficient to enable one to decide whether acetic acid (or a related intermediate) forms the sole precursor for cholesterol, because it is not known to what extent the dietary deuterio acetate was diluted by acetic acid formed in the organism. At present, even the occurrence of acetic acid as such in intermediary metabolism is not definitely established. However, the animal organism is undoubtedly capable of metabolizing acetic acid. The following discussion therefore refers not only to acetic acid itself but also to closely related compounds into which acetic acid might be converted by the animal.

The minimum fraction of cholesterol derived from dietary acetate can be estimated. The deuterium concentration in the cholesterol isolated from Experiment A (Table I) amounted to 1.3 per cent of that of the administered sodium acetate. Since the cholesterol analyzed represented a mixture containing approximately one-fifth of the cholesterol newly formed during the experimental period, the percentage of hydrogen derived from acetate during this period must have been $5 \times 1.3 = 6.5$ per cent. Furthermore, we know from previous findings that one-half of the total hydrogen atoms in cholesterol had, during its synthesis, originated from the body fluids. The body water in this experiment contained only 0.04 per cent deuterium. Since the deuterium concentration of the hydrogen derived from the body fluids could not have had a measurable value (<0.005 atom per cent excess), the deuterium concentration of the cholesterol hydrogen derived from acetate must have been twice as high as 6.5 per cent. We may, therefore, conclude that a minimum of 13 per cent of all the hydrogen in the newly formed cholesterol derived from organic molecules had originated in the dietary deuterio acetic acid.

Data on acetic acid production in the rat are not available. Bernhard (19), working on sulfanilamide acetylation, observed an approximately 10-fold dilution of deuterio acetate in rabbits. It may be inferred that, if acetic acid is formed at comparable rates in the rat and in the rabbit, the deuterio acetate or related compound would have been diluted several times by the ordinary analogue before being used for cholesterol synthesis. On this basis, a substantially higher fraction than the calculated 13 per cent of the cholesterol would have been derived from acetic acid. If acetic acid is the sole precursor of cholesterol, a large daily synthesis of acetic acid to dilute the dietary acetic acid must be postulated.

[4] MacLean and Hoffert (18) in their study on the formation of fat and sterols by aerated yeast observed an inhibition of sterol synthesis by sulfite, whereas it had no effect on fat formation. They account for the inhibition by suggesting that an aldehyde is an active metabolite in sterol synthesis.

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The deuterium concentrations in cholesterol after both α,β- and β,γ-dideuterio butyrate are fed are slightly higher than that of the body fluids; the deuterium of the cholesterol, therefore, cannot have been derived only from the body fluids. The results may be due to hydrolysis of acetoacetate to acetic acid, although the actual value indicates that under our experimental conditions hydrolysis of acetoacetic acid could not have been its main metabolic pathway.

The concentration of deuterium in the cholesterol of animals fed succinate seems too high to have been derived only from the body fluids. A slight conversion to acetic acid may have occurred. The essentially negative results obtained with propionic, succinic, and the butyric acids indicate that the conversion of acetate to cholesterol is a specific reaction scarcely shared by the related lower fatty acids.

The observation that in animals which had formed deuterio cholesterol from deuterio acetate the fatty acids had not taken up stably bound deuterium makes it seem unlikely that fatty acids were utilized. These results do not exclude the possibility that the carbon atoms of acetic acid are utilized for fatty acid formation. The mechanisms for these two syntheses, however, must be fundamentally different.[1]

In this connection it is of interest to consider the data reported by Sonderhoff and Thomas (20) on sterol formation by oxygenated yeast with deuterio acetate as the sole nutrient. Under their experimental conditions the saponifiable fraction of yeast contained roughly one-third as high a deuterium concentration as the deuterio acetate used. The acetate hydrogen did not exchange in the yeast culture and acetic acid was not formed by the cells, as the acetate recovered from the culture contained an unchanged isotope concentration. The deuterium content of the sterols was twice as high as that of the yeast fats and 20 times higher than that of the yeast carbohydrates. These authors interpret their data to indicate a rather direct utilization of acetate molecules for sterol synthesis.

A mechanism involving the condensation of a great number of acetic acid or related molecules accompanied by reduction of double bonds and uptake of hydrogen from the surrounding fluids would be in agreement with the results of Sonderhoff and Thomas for yeast as well as ours for the animal tissues. The preformed chains of the higher fatty acids, such as palmitic, stearic, or the corresponding unsaturated acids, have frequently been discussed as probable building stones for the sterol molecule (21). The evidence secured from the study of fat metabolism with the aid of deuterium points strongly against a participation in sterol synthesis of the higher fatty acids *per se*. Fecal sterols of rats which had received for 8 days an addition of deuterio palmitate to a sterol-free diet did not contain an excess of isotope (22). As the excreted sterols must have been derived from choles-

terol synthesized in the animal, the fatty acid as such could not have served as a precursor. The possibility that fatty acids are the source of acetic acid and thereby supply carbon and hydrogen for cholesterol synthesis still would not permit us to regard them as precursors in the accepted sense.

SUMMARY

1. The feeding of sodium deuterio acetate to mice and rats leads to the formation of deuterio cholesterol. By degradation of the sterol isolated from the animals, isotope was shown to be present in both the side chain and the nucleus of the cholesterol molecule.

2. A minimum of 13 per cent of the hydrogen atoms of cholesterol was derived from the acetate ion. The actual value must be higher, as the dietary acetate must have been diluted either by endogenous acetate or a closely related derivative into which the acetic acid is converted by the organism prior to utilization for sterol synthesis.

3. The experimental results exclude propionic, butyric, and succinic acids directly, and pyruvic and acetoacetic acids indirectly, as intermediates in the acetate-sterol conversion.

4. The absence of deuterium in the fatty acids of animals fed deuterio acetate is additional support for the previously expressed view that fatty acids are not directly involved in cholesterol synthesis.

BIBLIOGRAPHY

1. Bills, C. E., *Physiol. Rev.*, **15**, 23 (1935).
2. Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **121**, 235 (1937).
3. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **143**, 297 (1942).
4. Wieland, H., and Sonderhoff, R., *Ann. Chem.*, **499**, 213 (1932).
5. Swendseid, M. E., Barnes, R. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 47 (1942).
6. Leloir, L. F., and Muñoz, J. M., *Biochem. J.*, **33**, 731 (1939).
7. Krebs, H. A., and Johnson, W. A., *Biochem. J.*, **31**, 772 (1937).
8. Loeb, A., *Biochem. Z.*, **47**, 118 (1912).
9. Morehouse, M. G., *J. Biol. Chem.*, **129**, 769 (1939).
10. Mauthner, J., and Suida, W., *Monatsh. Chem.*, **17**, 41 (1896).
11. Bergmann, E., and Bergmann, F., *J. Chem. Soc.*, 1919 (1939).
12. Halford, J. O., and Anderson, C. L., *J. Am. Chem. Soc.*, **58**, 736 (1936).
13. Rittenberg, D., Schoenheimer, R., and Evans, E. A., Jr., *J. Biol. Chem.*, **120**, 503 (1937).
14. Fichter, F., and Sonneborn, F., *Ber. chem. Ges.*, **35**, 938 (1902).
15. Fischer, E., and Mouneyrat, A., *Ber. chem. Ges.*, **33**, 2387 (1900).
16. Schoenheimer, R., *J. Biol. Chem.*, **110**, 461 (1935).
17. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).
18. MacLean, I. S., and Hoffert, D., *Biochem. J.*, **20**, 343 (1926).
19. Bernhard, K., *Z. physiol. Chem.*, **267**, 91 (1940).
20. Sonderhoff, R., and Thomas, H., *Ann. Chem.*, **530**, 195 (1937).
21. Eckstein, H. C., and Treadwell, C. R., *J. Biol. Chem.*, **112**, 373 (1935-36).
22. Stetten, D., Jr., and Schoenheimer, R., *J. Biol. Chem.*, **133**, 317 (1940).

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THE CHEMISTRY OF EDIBLE RHUBARB

By H. A. W. BLUNDSTONE and D. DICKINSON

A plan of investigations into the chemical composition of rhubarb is described. Illustrations show the severe action which this product has on tinplate, and this is of great importance to the canning industry. The determination of the acid constituents of a number of varieties of rhubarb has shown that there is only a limited number of acids present and that all varieties contain the same major acids. There are differences in the concentrations of the acids according to season and age of growth. Oxalic acid appears to accumulate, probably as calcium and magnesium salts.

(I) Plan of investigations and methods used

Introduction

The composition of common rhubarb has seldom been the subject of scientific investigation of any kind and then mainly in connexion with cases of alleged poisoning. It is popularly supposed that rhubarb contains oxalic acid as a major constituent, although Augerhausen[1] pointed out in 1920 that malic acid is the predominant acid. Cases of poisoning have usually been associated with the consumption of the leaves of rhubarb which have been reported to contain as much as 1·1% of oxalic acid.[2] The soluble and total oxalate content of several varieties was determined by Mathot & Doesburg.[3]

Rhubarb is eaten as a fruit, being of comparatively high acidity for vegetable matter,

and only the leaf petioles are used. It is necessary to distinguish two forms of rhubarb even at this stage—'forced' rhubarb, and natural field or garden rhubarb. Forced rhubarb is grown under artificial conditions in the dark and is a valuable winter crop. Field or garden rhubarb grown under natural illumination is a much larger and coarser growth used mainly for industrial conservation and it is this form with which the present investigation is primarily concerned. It is an important crop, canned rhubarb being popular in Great Britain to the extent of about 9000 tons per annum (net can contents), of which almost one half is supplied in large cans to bulk caterers, bakers, and similar consumers. In addition large quantities of rhubarb are sold on the fresh market.

Canned rhubarb exhibits some peculiar properties. Because of its high acidity, it has always been deemed prudent to pack it in lacquered cans, since its natural pink colouring matter would form an unpleasant blue tin compound if plain cans were used. Internally lacquered cans of a quality satisfactory for other fruits are, however, often attacked by rhubarb, which has the facility of penetrating the lacquer, attacking the underlying tin-plate, and then destroying the adhesion of the lacquer to the tinplate. Severe cases have been seen in which the whole lacquer film has been detached. Modern improvements in lacquer formulations and in methods of application combine to reduce the severity of the effects of canned rhubarb, but it nevertheless remains a problem in comparison with other fruits. The constituent that is responsible for the peculiar corrosiveness of rhubarb has not been identified, but it is known to be a function of variety, some varieties being apparently free from this undesirable characteristic.

Rhubarb varieties display a remarkable range of colours—from pale green to beetroot-red in the extremes, with intermediate combinations of coloured or green flesh and skin in infinite variety. There appears to be little or no information about the chemistry of the colouring matters involved. From the industrial point of view it is highly desirable that the nature of the colouring matters and their stability under processing conditions should be known as fully as possible. Current practice is to add artificial colour to canned rhubarb and this is most successful with varieties from which the natural colouring matters—particularly the greens and browns—are absent. With such a range of naturally coloured materials available there is every likelihood of at least one variety being suitable for processing without the addition of artificial colouring matter.

Collections of rhubarb varieties ('Rhubarb Museums') are established at Stockbridge House Experimental Horticulture Station, Cawood, Yorks., and at the Royal Horticultural Society's Gardens at Wisley. The former collection comprises about 90 distinct varieties and it is with this one that all the preliminary work has been carried out. It is obvious that a complete investigation of so many varieties must take a considerable time and in order that the two industries—horticulture and canning—shall benefit from the early results, a number of varieties showing promise in regard to their growing characteristics have been selected for priority investigation. Samples of such varieties have been inspected and their pigmentation noted, then preserved by heat in glass jars with and without skin, for subsequent analysis. Other samples have been preserved by canning, without added colouring matter, and the quality of the canned rhubarb assessed. The corrosion of the cans by the rhubarb varieties has also been assessed.

Experimental

Preservation of samples for analysis

Bottling.—The sticks of rhubarb were divided into two portions; the outer skin (peel) was removed from one portion. They were then cut into approximately 1-in. lengths; large sticks were divided longitudinally also. Weighed quantities were filled into glass jars so as to fill the jars tightly, then covered with hot water, the caps fitted, and the jars sterilised by immersion in boiling water for a period appropriate to the size of jar. After being sealed and cooled, the samples were stored in the dark at 0–2° until required.

Canning.—The sticks were prepared in the same way as for bottling, then filled into cans appropriately lacquered internally, covered with syrup of 40° Brix, and processed in boiling water. The finished cans were stored at room temperature until required for examination.

Examination of canned samples

A double-purpose examination was carried out:

(i) to assess the quality of the rhubarb, involving its colour, texture, and flavour;
(ii) to assess the corrosion of the can.

The rhubarb was assessed on a simple comparative basis, with the additional recording of especially desirable or undesirable characteristics. Corrosion of the can was assessed by a method that has been used for many years in these laboratories.

(a) *Assessment of corrosion of cans*

The inside of the can is divided into three regions which may be scored separately. These regions are the end-seam, being the seam made by the can manufacturer, the side seam, and the body of the can. Each region is examined for three features—corrosion (or the attack on the tin surface), blistering of the lacquer coating, and staining of the surface. The scoring is on a numerical basis and the full system is shown in Table I.

Table I

Key to scoring of lacquer tests

Score	Corrosion	Blisters	Stains
1	Very slight; just visible on close inspection, but significant	One or two blisters about ¼ in. dia.	Faint; just seen on close inspection
2	Slight; noticeable without close inspection	One about ¼ in. dia. or two smaller	Just seen without close inspection
3	Moderate; not serious	One about ½ in. or two or three smaller	Distinctly noticeable
4	Rather serious and obvious to public	One about ¾ in. or two medium, or several smaller	Rather serious; obvious to public
5–6	Serious; just sufficient for public complaint	One about 1–1½ in. or many small	Serious; just sufficient for public complaint
7–9	Very serious	Very severe general blistering	Very serious
10–12	Up to complete stripping of plate		

Two cans which have been scored for corrosion are shown in Fig. 1. Both were normal rhubarb-lacquered cans and were opened after 3 years' storage. Can A contained rhubarb var. Prince Albert and shows very mild attack, whereas can B contained rhubarb var. Timperley Early and shows very severe corrosion. The results of the corrosion assessment on these two cans were:

Can A	End seam 1	Side seam 3	Body 2
Can B	" 4	" 8	" 8

No staining was observed. In this instance blistering had given rise to corrosion beneath the lacquer and was not assessed separately.

(b) *Quality assessment*

In the seasons 1960–61, 23 varieties were examined. The most suitable varieties were selected after canning and examination of the can contents for colour, texture and flavour. Six varieties were selected for further trials (marked * in Table II), mainly on the basis of colour.

These six varieties, together with a further six untested varieties, are being grown for a bulk pulling trial at the Stockbridge House Experimental Horticulture Station. In this instance the weight of the crop from a given size plot will be recorded, and vigour will be included in the assessment of these varieties in a later communication.

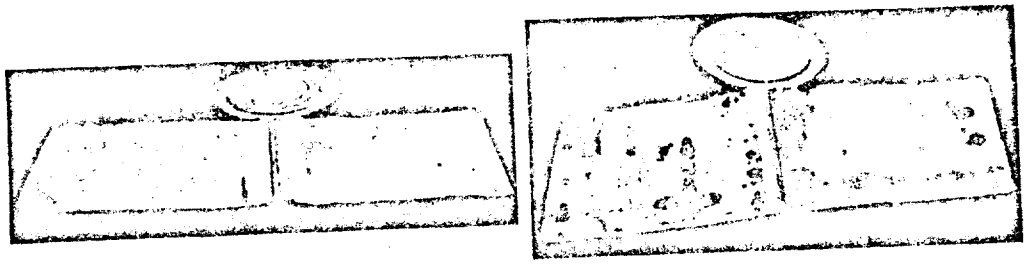

FIG. 1.—*Appearance of cans of rhubarb after 3 years' storage*
A (left) var. Prince Albert B (right) var. Timperley Early

Analysis of samples for acid content

An alcoholic extract of the acids was prepared and analysed by chromatography on a silica gel column by a method based on the gradient elution technique of Wager & Isherwood.[4] The method was slightly modified to suit the particular purpose and the changes are given below. To facilitate comparison with the original method the same subheadings have been used as in the original paper.

(i) *Reagents*

Silica gel.—Mallinkrodt's 'A.R. Silicic Acid 100 mesh' was employed. This was washed with water several times to remove finer particles.

Silica gel–10N-sulphuric acid mixture.—Silica gel was mixed with half its weight of 10N-sulphuric acid.

(ii) *Extraction*

The samples were preserved in water in jars and contained about 60% fresh weight of rhubarb. The whole sample was macerated and about 25 g. weighed into a beaker. This was again macerated after addition of 50 ml. of 95% ethanol and set aside for 2 h. The liquid was filtered off and the solid residue extracted with three further portions of 80% ethanol. The extracts were combined and made up to 250 ml.

Table II

Quality assessment of rhubarb varieties

Variety	Colour	Flavour	Texture
*Prince Albert	Green	Good	Good
Reed's Early Superb	Green–brown	Slightly sharp	,,
New Variety	Brown purple	,, ,,	,,
S.10	Green–brown	,, ,,	Fair
German Wine	,, ,,	Good	,,
*S.1 (Merton Foremost)	Green	Fair	,,
*Timperley Early	Green–brown	,,	Good
Magnum Bonum	Green	Good	Stringy
Amerikanske Kaempe	,,	Fair	Slightly tough
*Canada Red	Brown purple	,,	Fair
Appleton's Forcing	Green–brown	Weak	,,
Elmsfeuer	Pink purple	Fair	Stringy
Vroege Engelse	Green brown	,,	Fair
Buck's Early Red	Brown	Sharp	,,
Crimson Perfections	Pink brown	,,	,,
Tobolsk	Green–brown	,,	Tough
*Ruby	,, ,,	Good	Stringy
Edina	,, ,,	Sharp	Fair
Early Sunrise	,, ,,	Fair	Tough
Hobday's Giant	Green	,,	,,
S.12	Green brown	,,	,,
Vinrabarber Svenborg	Beetroot red	,,	Fibrous
*Victoria	Green brown	Rather acid	Fair

(iii) *Total acid determination*

A portion of macerate was used to determine total acidity by direct titration.

(iv) *Purification*

Fifty ml. of extract were taken and neutralised to phenolphthalein before evaporation to dryness, to prevent loss of volatile acids. The residue was dissolved in 4 ml. of water and 8 g. of silica gel were added. The procedure followed was the same as that of Wager & Isherwood except that washing with light petroleum was not found necessary.

If volatile acids are not required, acidification of the gel to pH 1 can be carried out before addition to the column.

(v) *Separation and analysis*

Two columns only were used for the analysis. The upper column (Quickfit & Quartz 18 × 400 mm.) was packed with 7 g. of silica gel mixed with 3·5 ml. of 0·75M-sodium sulphate in 0·5N-sulphuric acid. The lower column (Quickfit & Quartz 10 × 300 mm.) was packed with 7 g. of silica gel mixed with 3·5 ml. of 0·5N-sulphuric acid. Filter paper discs were placed at the upper and lower ends of each column.

The acid mixture in 0·35 ml. of water was mixed with 0·7 g. of silica gel and added to the top of the lower column.

About 120 fractions of 3 ml. were collected in test tubes during 7 h. It was found necessary to add a further 1 ml. of ethanolic phenolphthalein to the early tubes in order to prevent the separation of aqueous droplets. Air was not bubbled through the tubes before titration.

(vi) *Identification*

The acids were identified by paper chromatography. After titration, the samples constituting a single peak were concentrated and passed through a small column (0·5 g.) of a cation-exchange resin (Zeo-carb 215).

Volatile acids were identified by the method of Mukerjee.[5] For non-volatile acids, two-dimensional chromatography with n-propanol-ammonia solution (sp. gr. 0·88) (7 : 3) and butyl formate-formic acid-water (10 : 4 : 1) was used.[6]

The sequence of elution of the acids is : peak 1, acetic acid ; peak 2, unknown(s) ; peak 3, succinic + lactic acids ; peak 4, oxalic acid ; peak 5, probably glycollic acid ; peak 6, malic acid ; peak 7, citric acid.

(vii) *Recovery of acids*

A solution was prepared by dissolving accurately weighed quantities of acids in water and separating these on the column by the standard method. The results obtained by analysis were compared with the weights taken and these showed that the method was sufficiently accurate for the acids examined (Table III, section A).

As a check on the purification procedure, two samples of a rhubarb extract were taken. One of these was analysed for the acid content, and to the other were added known weights of four acids found to be present. This was then analysed and the increased quantities found corresponded with the amounts added (Table III, section B).

(II) Quantitative analysis of acid constituents

The same sequence of acids has been found in all of the rhubarb varieties so far examined, but quantitatively there is large variation between varieties. The acids of var. Canada Red (see Table VIII) are shown graphically in Fig. 2.

Attempts have been made to determine whether the variation in acid content is due to sampling errors or whether it reflects a true varietal characteristic. The variety Timperley Early was sampled and analysed in the fresh state. Three different petioles were taken, two from the same plant, and the third from a different plant (labelled A, B and C, respectively, in Table IV). The upper and lower halves of one petiole were analysed separately ; the other two were macerated and analysed. Samples D and E (Table IV) were taken at different times

Table III

Recovery of acids by chromatography

Acid	A recovery from standard mixture			B recovery from rhubarb + standard mixture				
	Wt. taken, mg.	Wt. recovered, mg.	% recovered,	Wt. originally present, mg	Wt. added, mg.	Expected total	Total found	% recovered
Acetic (A.R.)	2·0	2·1	105	—	—	—	—	—
Succinic	1·0	1·1	110	0·1	3·0	3·1	2·8	91
Oxalic (A.R.)	3·5	3·4	97	5·7	3·5	9·2	9·3	101
Malic*	30·0	27·0	90	43·6	4·9	48·5	50·0	103
Citric (A.R.)	5·5	5·4	98	4·0	6·6	10·6	10·1	95

* very impure sample

and bottled by the standard procedure. It is evident that the quantity of each acid present varies considerably within the single variety, indicating that the positive distinction between two varieties on the grounds of acid constituents is likely to prove difficult without undertaking a vast number of analyses. However, there was no indication of a big difference in content between the upper and lower halves of a single petiole.

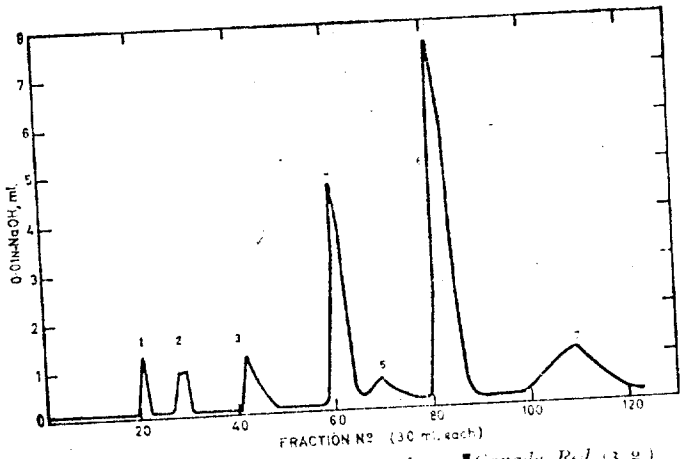

FIG. 2.—*Acids separated from rhubarb var. Canada Red (3/8)*

Effect of time of sampling

Several petioles were collected from the same plant (Victoria Seedling variety) at intervals of 4 weeks, the sample being bulked for analysis on each occasion. The results are tabulated in Table V. Here again, very considerable variations are apparent which may reflect the

Table IV

Acid analysis of samples of var. Timperley Early

(results expressed as mg. per g. fresh wt.)

Acid	Sample A Upper half	Sample A Lower half	Sample A Total	Sample B	Sample C	Sample D	Sample E
Acetic	0·45	0·49	0·47	0·45	0·33	1·35	0·10
Acid β	0·29	0·26	0·27	0·10	0·14	0·88	0·32
Succinic + lactic*	0·39	0·31	0·35	0·15	0·20		
Oxalic	1·75	1·20	1·45	1·05	1·09	1·34	1·81
Malic	10·4	9·3	9·9	13·0	12·0	14·4	0·3
Citric	0·72	0·50	0·61	N.A.	N.A.	1·15	N.A.

* Where results are given for succinic and lactic acids, the calculation is based on the assumption that the two acids are present in equal amounts

Table V

Acid analyses of var. Victoria Seedling sampled over a period

(results expressed as mg. per g. fresh wt.)

Acid	Week 1	Week 5	Week 9	Week 13
Acetic	0·64	1·05	0·78	0·68
Acid *β*	0·02	0·97	0·17	0·48
Succinic lactic*	0·31	0·19	0·13	0·09
Oxalic	1·52	2·15	3·03	3·25
Malic	12·7	13·5	11·0	16·6

* Where results are given for succinic and lactic acids, the calculation is based on the assumption that the two acids are present in equal amounts.

sampling errors. However, oxalic acid increased steadily over the period. It should be pointed out that the petioles taken as samples on any occasion would not necessarily be of an age measurable by the sampling time. They were all healthy growths, but those taken on the thirteenth week might well have developed at any time during the period. This complication is one demanding a special labelling and sampling technique for its avoidance.

Total (acid-soluble) anions

The extraction procedure described allows the determination of soluble (generally 'uncombined') acid only. In view of the apparent fixation of oxalic acid, it was thought necessary to determine the total or acid-soluble anions. For this purpose macerated 25-g. samples were extracted with 50 ml. of 95% ethanol; then with 50 ml. of 95% ethanol + 5 ml. of conc. hydrochloric acid; then twice more with 50 ml. of 80% ethanol + 2·5 ml. of conc. hydrochloric acid; and the four extracts were combined. The soluble and acid-soluble organic acids in four varieties are given in Table VI. It is apparent that oxalic acid is the only acid of which a

Table VI

Soluble and acid-soluble organic acids in four varieties of rhubarb

(results expressed as mg. per g. fresh wt.)

Acid	Merton Foremost (S1)		Canada Red		Timperley Early (1961)		Timperley Early (1962)	
	Soluble	Total	Soluble	Total	Soluble	Total	Soluble	Total
Acetic	0·43	0·92	0·40	1·36	1·35	1·34	0·46	0·92
Acid *β*	0·63	0·64	0·48	1·20	0·88	0·88	0·40	0·40
Oxalic	1·53	2·67	2·71	4·80	1·34	2·18	0·83	1·24
Malic	12·40	11·70	11·90	11·80	14·40	13·80	10·55	9·38
Citric	0·67	0·68	1·00	1·10	1·15	1·34	1·20	1·17
Sol. oxalate as % of total oxalate	57·5		50·5		61·4		67·0	

large proportion is consistently insoluble in water, and the constancy of this proportion is quite remarkable. Analyses have been carried out for calcium and magnesium and, as Table VII shows, the equivalent quantities of calcium and magnesium oxalates correspond reasonably well with the quantities of water-insoluble oxalate.

Table VIII summarises the acid contents of a number of varieties of rhubarb sampled and preserved as previously described.

Table VII

Insoluble oxalate in rhubarb varieties

(results as mequiv. per 100 g. fresh weight)

Variety	Merton Foremost	Canada Red	Timperley Early
Insoluble oxalate	2·5	4·65	1·86
Calcium	1·75	4·65	1·75
Magnesium	0·66	0·06	0·57
Calcium + magnesium	2·41	5·31	2·32

Conclusions

The major acids that occur in rhubarb are acetic, an acid termed β in the present report (thought most likely to be fumaric), succinic, lactic, oxalic, malic and citric. Of these, malic acid predominates in all varieties. There is evidence that oxalic acid accumulates in the leaf petioles and almost one half of it is not soluble in water. This portion is probably combined as calcium and magnesium salts.

There is at least as great a variation in the contents of individual acids between samples of the same variety as there is between samples of different varieties and, although two of the samples analysed, Edina and German Wine, had exceptionally low oxalic acid contents it is not possible at this stage to infer that this is a varietal characteristic.

Although the varieties so far examined show a considerable range of corrosiveness, the acid content varies to a lesser extent. It is thought likely that factors other than acidity influence the corrosion.

Table VIII

Variety	Acid content mg. per g. fresh wt.					Total acidity	
	Acetic	β	Oxalic	Malic	Citric	as ml. 0·01 N-NaOH per g.	as acetic acid, mg./g.
New Variety	0·32	0·45	1·50	13·00	1·80	26·3	15·8
Sto	0·23	0·13	1·63	11·80	1·31	24·1	14·5
Edina	0·90	0·38	0·73	9·00	1·00	20·9	12·5
Timperley Early	0·19	0·32	1·81	9·30	0·80	23·0	13·8
Magnum Bonum	0·34	*	1·90	9·20	1·13	21·5	12·9
Amerikanske Kaempe	1·58	*	1·47	8·80	1·53	22·5	13·5
Ruby	0·24	0·22	1·53	8·00	1·80	19·8	11·9
Prince Albert	0·79	*	1·27	8·90	1·19	19·5	11·7
Canada Red	0·46	0·20	1·93	8·90	1·53	21·8	13·1
German Wine	0·56	0·51	0·64	9·60	1·68	20·8	12·5
Appleton's Forcing	0·65	0·36	1·08	8·60	2·00†	20·9	12·5
Elmsfeuer	0·38	0·22	1·18	11·00	1·55	23·3	14·0
S1 (Merton Foremost)	0·48	0·06	1·46	10·20	0·97	21·1	12·7
Vroege Engelse	0·48	0·54	1·68	10·40	0·97	23·4	14·0
Holiday's Giant	0·72	0·23	1·28	10·70	1·60	23·2	13·9
Crimson Perfections	0·63	0·27	1·78	11·70	0·77	23·9	14·3
Reed's Early Superb	0·35	0·10	1·47	11·50	1·00	—	—
Early Sunrise	1·28	1·03	1·24	12·00	1·23	28·5	17·1

* Included with acetic acid † By difference

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References

[1] Augerhausen, *Z. Unters. Nahr.-Genussm.*, 1920, **39**, 81
[2] Van Itallie, & Lemkes, *Pharm. Weekbl.*, 1917, **54**, 1234
[3] Methot, H. J. & Doesburg, J. J., *Voeding*, 1950, **11**, 123
[4] Wager, H. G. & Isherwood, F. A., *Analyst*, 1963, **88**, 200
[5] Mukerjee, H., *Analyt. Chem.*, 1959, **31**, 1284
[6] Blundstone, H. A. W., *Nature, Lond.*, 1963, **197**, 377

Behavior of Free Acetate in Miniature Swine.

Acetate Metabolism in Miniature Swine

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Summary

1. The blood acetate level has been followed over the course of 8 months in a group of 16 miniature swine kept under standard conditions.

2. The mean acetate level was 195 nmoles/ml plasma, about double that found in man. A high fat diet did not affect this value and animals kept in stalls, or in the open gave identical results.

3. Acetate thiokinase and acetyl CoA deacylase were present in all tissues tested; the activities were similar to those of the rat.

4. A particle free extract of aorta was capable of incorporating acetate into long chain fatty acid at a rate similar to that found in the rat aorta. The significance of these findings for the development of atherosclerosis is discussed.

Key-Words: Miniature swine - Acetate metabolism - Blood acetate - Atherosclerosis.

Introduction

Miniature swine frequently develop atheromatous vascular changes which, from a pathological-anatomic viewpoint, are very similar to those observed in man. In connection with a study carried out by several working groups on the suitability of miniature swine as a model for the formation of arteriosclerosis, we have investigated the acetate metabolism on this experimental animal.

The behavior of plasma acetate appears to play an important role in the lipoid metabolism of vascular walls (Hochheuser *et al.*, 1964; H. Weiss, 1967). In a supplement to studies which confirmed the incorporation of C^{14}-acetate into fatty acids and cholesterol of the intact rat aorta, the synthesis of fatty acids from acetate and acetyl-CoA also in particle-free aorta extracts could be established. In the intact rat aorta, the *in vitro* synthesis of fatty acids depends upon the acetate concentration (Hochheuser *et al*,). At concentrations corresponding to the normal blood acetic acid level, fatty acid synthesis takes place at about the half-maximum rate, and thus would appear to be influenced also *in vivo* by the amount of available acetate. In rats with chronic diabetes, the acetic acid level in blood increases, and also in man after acute alcohol exposure. The possible significance of these processes for atheromatosis was investigated on miniature swine.

The steady-state blood acetate level represents the resultant from the acetate formation, acetate consumption and possibly also acetate loss through the urine. The latter could not yet be investigated so far. The following diagram, $\text{Acetyl-CoA} \underset{\text{ATP}}{\overset{H_2O}{\rightleftharpoons}} \text{Acetate} + \text{CoA}$ for the formation of free acetate in animal tissues has been described by Hepp *et al.* (1966). As a result, in animals which do not feed on plants, acetate should originate mainly from acetyl-CoA. According to this diagram, the two enzymes acetate thiokinase (E.6.2.1.1.) and acetyl CoA deacylase (E.3.1.2.1.) control the formation and cleavage (splitting) of that acetyl-CoA portion which is not immediately converted in the citric acid cycle or is used for other reactions, such as fatty acid synthesis, etc. An indication of the validity of such an "acetate cycle" was provided by tests with Ehrlich ascites cells (Hepp *et al.*). The determination of both of these enzymes in the liver and other organs of miniature swine was there-

fore an important part of the studies reported here. The final section of this report is concerned with studies of the fatty acid-synthesizing enzyme system in the aorta of miniature swine.

Methods

Test animals. Miniature piglets born from 3 different mothers were weaned for 2 months and divided into 2 groups of 8 piglets each. One group was fed the usual commercial diet, and the other group received 10% additional fat (see Table 2 for the composition of the fat diet). Each group was further subdivided into 2 subgroups of 4 piglets each, whereby one subgroup was kept in stalls in a closed space and the other in huts in the open (cf. Kalich *et al.*).

Preparation of Material for Study

a) Blood (10-12 ml) was obtained by puncture of the jugular vein and transferred to centrifuge glass tubes containing one drop of a heparin solution. After mixing, the blood was immediately centrifuged (2000 g, 20 min) and aliquot portions of the plasma were collected with a pipet. Protein removal from the plasma was done as rapidly as possible by adding perchloric acid in an end concentration of 3%. The protein-free supernatant was adjusted to pH 3 with NaOH and 1M citrate buffer and subjected to microdistillation (Bartley) for the determination of acetate.

Distribution of acetate between plasma and corpusclar blood fractions: 33% of the acetate was present in the plasma, and 67% in the corpuscular fraction. When exogenous acetate was added to whole blood, a complete equalization (partition) between both components did not take place after 30 min; from this fact, we can conclude that equalization (partition) be-

tween the two compartments only takes place at a relatively slow rate. Heparinized plasma, isolated immediately after blood collection, was used in the following series of tests.

b) Tissue. The liver, kidneys, aorta and heart muscle were removed as rapidly as possible from the animals, which were killed by intracardial injection of Nembutal. The organs were immediately thrown into liquid nitrogen, and then ground in a mortar under steady addition of liquid nitrogen. The powders were extracted with Krebs-Henseleit buffer (1: 5) and centrifuged for 30 min at 100,000 g. The clear supernatants were used for the enzyme determinations.

Acetate Determination

a) Isolation of acetate from plasma. Microdistillation according to Bartley was used instead of the previously described Conway method (Hepp *et al.*). This method works much more rapidly and requires only 1 hour for complete distillation, in contrast to the 36 hours required in the Conway method. The flask of the distillation vessel contained Hg water-free Na_2So_4; 1 ml of protein-free plasma was uniformly distributed with a pipet over the surface of the powder. After evacuation, the vessel was incubated, according to Bartley's instructions, whereby the flask containing the sample was kept at 55°C, and the receiver at - 10°C. After 1 hour, 92% of the acetate present had passed over into the distillate.

b) The determination of acetate was carried out according to the procedure of Hepp *et al.*, based on the acetylation (proposed by Lundquist *et al.*) of sulfonamide with an enzyme extract from pigeon liver.

Enzyme Determinations

Acetate thiokinase and acetyl-CoA deacylase were determined in the 100,000 g supernatant according to the instructions of Hepp *et al*. The test for acetate thiokinase is based on the following coupled reactions:

$$C^{14}\text{-acetate} + \text{CoA} + \text{ATP} \xrightarrow{\text{Thiokinase}} C^{14}\text{-acetyl-CoA} + \text{AMP} + \text{pp}$$

$$C^{14}\text{-acetyl-CoA} + \text{oxalacetate} \xrightarrow{\text{Citr. synth.}} C^{14}\text{-citrate} + \text{CoA}$$

The C^{14}-citrate formed is precipitated as the silver salt, isolated and counted. Time and protein dependency tests gave linear calibration curves.

The acetyl-CoA- deacylase test is based on the discoloration of 2, 4-dichlorophenol-indophenol by CoASH, which is liberated during enzymatic cleavage from acetyl-CoA.

The incorporation of radioactive acetate into fatty acids by extracts from aorta tissue was measured by the method of Numa *et al*. Details of this method are described by Hochheuser *et al*.

Results and Discussion

Plasma Acetate Levels in Miniature Swine

a) Comparison with other animal species (Table 1). Miniature swine have a plasma acetate level of 192 nanomoles/ml, a value which is very close to that found in the domestic swine and in the rat. However, this level is at least twice as high as that of man. This fact could be explained as follows: plant-eating animals, such as, for example, the goat (Table 1), have an extremely high blood acetate level, which can be considered as the result of a direct acetate absorption from the intestine.

We should keep in mind the fact that both swine and rats, which are kept under laboratory conditions, receive a considerable amount of green fodder supplied with the food, and that this could cause a rise in the blood acetate level, corresponding to that observed in plant-eating animals.

Table 1. Comparison of the plasma acetate levels in different animal species. Acetate was determined in heparinized plasma by the method of Hepp *et al*. Comparison values for other animal species were taken from the studies of Hochheuser *et al*. and Lundquist *et al*.

Species	(1) Anzahl der Untersuchungen	Plasmaacetat (nmol/ml) (2)
(3) Ratte	26	195 ± 10
(4) Schwein (normal)	3	189 ± 6
(5) Miniaturschwein	108	192 ± 4
(6) Mensch	5	73 ± 20
Mensch (LUNDQUIST)		50—100
(7) (8) Ziege (MENAHAN)		1000—1200

1. Number of investigations
2. Plasma acetate (nanomoles/ml
3. Rat
4. Swine (normal)
5. Miniature swine
6. Man
7. Man (Lundquist)
8. Goat (Menahan)

b) Effect of nutrition and living conditions on the plasma acetate level (Table 2). Two points appear to be of interest here. First, the considerable variation (fluctuations) of the acetate level in the different animal groups. We do not consider it probable that these fluctuations can be ascribed to the method of determination; rather, it seems possible that a different degree of stress during the very radical blood collection procedure could be responsible for these variations. In comparison, the values obtained by Weiss for rats and by Lundquist for man are remarkably constant. In these tests, every kind of stress was excluded whenever possible. Thus, for example, rats were killed under particularly careful (sparing) conditions (personal communication from H. Weiss). As far as the avaliable data allow us to judge, neither a

special fat diet nor the different living conditions of the animals exerted a striking effect on the blood acetate level. Perhaps the result is not too surprising, since rats fed exclusively on lard also did not exhibit any changes in their blood acetate level (Weiss). It will be of interest, therefore, to follow the course of the blood acetate level in an advanced stage of fat feeding, i.e. in older animals in which the formation of arteriosclerotic vascular changes have already taken place. It will also be advantageous to switch to the method of gas chromatography, which can be handled with greater precision, for the quantitative determination of acetate.

Table 2. Plasma acetate level of miniature swine under different conditions. The swine (2 months old at the beginning of the test) were divided into 4 groups of 4 animals each: Groups I and II received a 10% special fat diet (1), while Groups III and IV received a normal feed. Groups I and III were kept in stalls, and Groups II and IV - in the open. Plasma acetate was determined in protein-free plasma as described above. Values are given in nanomoles/ml; average values ± SEM (?)

Date → Datum	Gruppe I	Gruppe II	Gruppe III	Gruppe IV
Oct. 20.(10) 1966	179 ± 45	139 ± 20	242 ± 62	78.S (2)
Nov. 3.(11) 1966	64 ± 14	136 ± 28	146 ± 33	116 ± 18
22.(11) 1966	170 ± 35	220 ± 44	184 ± 33	183 ± 23
Jan. 11.(1) 1967	308 ± 51	146 ± 61	383 ± 39	287 ± 56
Mar. 7.(3) 1967	221 ± 49	148 ± 43	205 ± 33	239 ± 51
Apr. 21.(4) 1967	214 ± 15	179 ± 30	300 ± 39	280 ± 41
June 1.(6) 1967	75 ± 17	84 ± 8	88 ± 22	103 ± 3
Average values of group: Mittelwerte der Gruppe	176 ± 34	150 ± 19	221 ± 43	184 ± 30

Group

[1] Die Fettsäurenzusammensetzung der Diät (Zöllner u. Tacconi): $C_{12:0}$ 0,3%; $C_{14:0}$ 1,6%; $C_{16:0}$ 55,6%; $C_{18:0}$ 25,8%; $C_{18:1}$ 14,0% and $C_{18:2}$ 2,7%.

Fatty acid composition of diet

Enzymes of Acetate Metabolism

The activity of acetate thiokinase and acetyl-CoA-deacylase in various tissures of miniature swine is listed in Table 3. An estimate of the potential acetate formation rate can be obtained from the deacylase/thiokinase ratio; at high ratios, a predominance of acetate formation can be expected (Hepp *et al.*). As can be seen from Table 3, the miniature swine does not differ significantly in this respect from the rat. The aorta could be an exception, although the very low enzyme activities should be interpreted with caution. The high dencylase/thiokinase ratio of 6.2 would bring to mind a primarily acetate-producting tissue. This point has not yet been checked. However, the next experiment shows that, in spite of this low thiokinase activity, a notable fatty acid synthesis from acetate is possible in crude aorta extracts.

Table 3. Thiokinase and deacylase activity in various organs of the miniature swine and rat. The enzymes were determined by the methods of Hepp *et al.* after hydrolysis of deep-frozen samples in the 100,000 g supernatant. The age of the miniature swine was 53 and 107 days, resp. Values are given in nanomoles/mg protein/min (in each case, average values ± SEM (?) from 3-4 determinations, and in rats from 4-6 determinations).

Organ	Miniaturschwein – Miniature swine			Ratte – Rat		
	Thiokinase	Deacylase	Deacylase / Thiokinase	Thiokinase	Deacylase	Deacylase / Thiokinase
Liver Leber	2,63 ± 0,98	8,78 ± 2,87	3.35	5,57	9.77	1,7
Kidney Niere	3,08 ± 1.22	5.89 ± 1.82	1.91	6,53	10,2	1,6
Heart Herz	4.78 ± 1,59	12.1 ± 3.17	2,54	1,75	1,6	0,92
Aorta	0,32 ± 0,08	1,99 ± 0.9	6,2			

Fatty Acid Synthesis from Acetate

A decisive test for the idea that the height of the blood acetate level and the onset of arteriosclerosis are related to each other is the detection (identification) of an enzyme system, capable of synthesizing fatty acids, in the corresponding tissue, namely the aorta. Table 4 shows that rather uniform rates of incorporation of C^{14}-acetate into fatty acids were found in the 3 young miniature swine tested. Table 4 shows further that the rate of fatty acid synthesis in swine aorta does not differ greatly from the rate found in rat aorta.

Table 4. Incorporation of C^{14}-acetate into fatty acids by a particle-free aorta extract of the miniature swine and rat. The C^{14}-acetate incorporation into fatty acids was measured by the method of Numa et al. The particle-free aorta extract was prepared according to Hochheuser et al. All values are given in nanomoles/mg protein/3hrs.

Miniature swine

Rat

	^{14}C-Einbau in Fettsäuren
Miniaturschwein	
I	0,030
II	0,020
III	0,029
Ratte (HOCHHEUSER) Mittelwerte von fünf Werten	0,035 ± 0,004

← C^{14} incorporation into fatty acids

Averages of 5 values

The half-saturation concentration for acetate, i.e. the acetate concentration at which the in vitro fatty acid synthesis takes place at a half-maximum rate, was determined for the rat aorta as $K_m = 1.5 \times 10^{-4}$M (Hochheuser et al.). Since the blood acetate level of the rat (Table 1) is equal to 1.95×10^{-4}M, it is possible, on the basis of enzyme-kinetic considerations, that fluctuations in the blood acetate level could have a noticeable effect on the rate of the fatty acid synthesis taking place in the aorta. In future tests, therefore, the K_m in this system will also be determined for the aorta of miniature swine.

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Bibliography

BARTLEY, W.: Biochem. J. 53, 87 (1953).
HEPP, D., E. PRÜSSE, H. WEISS u. O. WIELAND: Biochem. Z. 344, 87 (1966).
HOCHHEUSER, W., H. WEISS u. O. WIELAND: Z. klin. Chem. 2, 175 (1964).
KALICH, J., K. KRUMBACHER u. B. RUF: Z. ges. exp. Med. 145, 312 (1968).
LUNDQUIST, F., U. FUGMANN, E. KLÄNING, and H. RASMUSSEN: Biochem. J. 72, 409 (1959).
MENAHAN, L., Persönliche Mitteilung.
NUMA, S., M. MATSUHASHI u. F. LYNEN: Biochem. Z. 334, 203 (1961).
WEISS, H.: Dissertation. München 1967.
ZÖLLNER, N., and M. TACCONI: Natr. et Dieta (Basel) 10, 777 (1968).

Zeitschrift für die gesamte experimentelle Medizin 145, 346—352 (1968)

Über das Verhalten von freiem Acetat beim Miniaturschwein

Acetatstoffwechsel beim Miniaturschwein

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Eingegangen am 1. September 1967

Summary. 1. The blood acetate level has been followed over the course of 8 months in a group of 16 miniature swine kept under standard conditions.

2. The mean acetate level was 195 nmoles/ml plasma, about double that found in man. A high fat diet did not affect this value and animals kept in stalls, or in the open gave identical results.

3. Acetate thiokinase and acetyl CoA deacylase were present in all tissues tested; the activities were similar to those of the rat.

4. A particle free extract of aorta was capable of incorporating acetate into long chain fatty acid at a rate similar to that found in the rat aorta. The significance of these findings for the development of atherosclerosis is discussed.

Key-Words: Miniature swine — Acetate metabolism — Blood acetate — Atherosclerosis.

Zusammenfassung. 1. Bei einer Gruppe von 16 Miniaturschweinen, die unter Standardbedingungen gehalten wurde, wurde der Acetatspiegel im Blutplasma über einen Zeitraum von 8 Monaten verfolgt.

2. Der mittlere Plasmaacetatspiegel betrug 195 nMol/ml Plasma, d. i. etwa doppelt so hoch wie beim Menschen. Verfütterung einer fettreichen Diät hatte keinen Einfluß auf den Plasmaacetatspiegel. Ebenso ergaben sich keine Unterschiede bei Tieren, die im Stall gehalten wurden, oder die freien Auslauf hatten.

3. Die Enzyme Acetat-Thiokinase und Acetyl-CoA-Deacylase wurden in allen der untersuchten Gewebe angetroffen. Ihre Aktivität war derjenigen von Rattengeweben vergleichbar.

4. Partikelfreie Extrakte aus Miniaturschweineaorten vermögen Acetat mit etwa gleich großer Geschwindigkeit in langkettige Fettsäuren einzubauen wie Extrakte aus Rattenaorten. Die Bedeutung dieser Befunde für die Entwicklung degenerativer Gefäßwandveränderungen wird diskutiert.

Schlüsselwörter: Miniaturschwein — Acetatstoffwechsel — Blutacetat — Arteriosklerose.

Einleitung

Das Miniaturschwein entwickelt häufig atheromatöse Gefäßveränderungen, die pathologisch anatomisch denjenigen beim Menschen sehr nahe kommen. Im Zusammenhang mit einer von mehreren Arbeitsgruppen durchgeführten Studie über die Eignung des Miniaturschweines als Modell für die Entstehung der Arteriosklerose wurden bei uns Untersuchungen über den Acetatstoffwechsel bei dem Versuchstier durchgeführt.

Das Verhalten des Plasmacetats erscheint für den Lipoidstoffwechsel der Gefäßwand von Bedeutung (Hochheuser et al., 1964; H. Weiss, 1967). In Ergänzung zu Untersuchungen, die den Einbau von ^{14}C-Acetat in Fettsäuren und Cholesterin intakter Rattenaorten bestätigten, konnte die Fettsäuresynthese aus Acetat und Acetyl-CoA auch in partikelfreien Aortenextrakten nachgewiesen werden. In der intakten Rattenaorta ist die Fettsäuresynthese in vivo von der Acetatkonzentration abhängig (Hochheuser et al.). Bei Konzentrationen, die dem normalen Blutessigsäurespiegel entsprechen, verläuft die Fettsäuresynthese etwa mit halbmaximaler Geschwindigkeit und dürfte somit auch in vivo vom Acetatangebot her beeinflußt werden. Bei chronisch diabetischen Ratten steigt der Essigsäurespiegel im Blut an, ebenso beim Menschen nach akuter Alkoholbelastung. Die mögliche Bedeutung dieser Vorgänge für die Atheromatose wurde am Miniaturschwein untersucht.

Der steady-state-Blutacetatspiegel stellt die Resultante aus Acetatbildung, Acetatverbrauch und möglicherweise auch -verlust durch den Urin dar. Letzteres konnte bis jetzt noch nicht untersucht werden. Das vorliegende Schema

$$\text{Acetyl-CoA} \xrightarrow[\text{ATP}]{\text{H}_2\text{O}} \text{Acetat} + \text{CoA}$$

der Bildung von freiem Acetat in tierischen Geweben wurde von Hepp et al. (1966) beschrieben. Demzufolge würde bei Nichtpflanzenfressern Acetat vornehmlich aus Acetyl-CoA stammen. Nach diesem Schema kontrollieren die beiden Enzyme Acetat-Thiokinase (E. 6.2.1.1.) und Acetyl-CoA-Deacylase (E. 3.1.2.1.) die Bildung und Spaltung desjenigen Acetyl-CoA-Anteils, der nicht sofort im Citronensäurecyclus umgesetzt oder für andere Reaktionen, wie Fettsäuresynthese etc., verwendet wird. Einen Hinweis für die Gültigkeit eines solchen „Kreislaufs des Acetats" brachten Versuche mit Ehrlich-Ascites-Zellen (Hepp et al.). Die Bestimmung dieser beiden Enzyme in Leber und anderen Organen des Miniaturschweines stellte daher einen wichtigen Teil der hier vorgelegten Untersuchungen dar. Der letzte Abschnitt dieses Berichtes befaßt sich mit Untersuchungen über das Fettsäure synthetisierende Enzymsystem in der Aorta des Miniaturschweines.

Methoden

Versuchstiere. Miniaturschweinferkel von drei verschiedenen Müttern wurden 2 Monate gesäugt und in zwei Gruppen von je acht Schweinen geteilt. Eine Gruppe wurde mit der üblichen kommerziellen Diät gefüttert, wobei die andere zusätzlich 10% Fett erhielt (siehe Tabelle 2 für die Zusammensetzung der Fettdiät). Jede Gruppe wurde weiterhin in zwei Untergruppen von je vier Schweinen geteilt, eine Gruppe wurde im Stall im geschlossenen Raum, die andere in Hütten im Freien gehalten (vgl. Kalich et al.).

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Gewinnung des Untersuchungsgutes

a) Blut (10—12 ml) wurde durch Punktion der Jugularvene gewonnen und in Zentrifugengläser, die einen Tropfen Heparinlösung enthielten, übergeführt. Nach Durchmischung wurde das Blut unmittelbar zentrifugiert (2000 g, 20 min) und aliquote Teile des Plasmas abpipettiert. Die Enteiweißung des Plasmas erfolgte so rasch wie möglich im Eisbad durch Zugabe von Perchlorsäure in einer Endkonzentration von 3%. Der eiweißfreie Überstand wurde mit NaOH und 1 M Citratpuffer auf pH 3 eingestellt und der Mikrodestillation (Bartley) für die Acetatbestimmung unterzogen.

Verteilung von Acetat zwischen Plasma und corpusculären Anteilen des Blutes: 33% des Acetats fand sich im Plasma, 67% im corpusculären Anteil. Wurde exogenes Acetat dem Vollblut zugesetzt, so trat nach 30 min kein völliger Ausgleich zwischen den beiden Bestandteilen ein, woraus geschlossen werden kann, daß der Ausgleich zwischen den beiden Kompartimenten nur relativ langsam vor sich geht. In der folgenden Versuchsserie wurde heparinisiertes, sofort nach der Blutentnahme isoliertes Plasma genommen.

b) Gewebe. Leber, Niere, Aorta und Herzmuskel wurden so rasch wie möglich von den Tieren entnommen, die durch intrakardiale Injektion von Nembutal getötet worden waren. Die Organe wurden sofort in flüssigen Stickstoff geworfen und anschließend unter ständiger Zugabe von flüssigem Stickstoff in einem Mörser pulverisiert. Die Pulver wurden mit Krebs-Henseleit-Puffer (1:5) extrahiert und dann 30 min bei 100000 g zentrifugiert. Die klaren Überstände wurden für die Enzymbestimmungen eingesetzt.

Acetatbestimmung

a) Abtrennung des Acetats aus dem Plasma. An Stelle der früher beschriebenen Conway-Methode (Hepp et al.) wurde die Mikrodestillation nach Bartley verwendet. Diese Methode arbeitet wesentlich rascher und benötigt nur 1 Std zur vollständigen Destillation im Gegensatz zu den 36 Std, die bei der Conway-Methode notwendig sind. Der Kolben des Destillationsgefäßes enthielt 4 g wasserfreies Na_2SO_4. 1 ml des enteiweißten Plasmas wurde gleichmäßig mit einer Pipette auf die Pulveroberfläche verteilt. Nach Evakuieren wurde das Gefäß entsprechend der Vorschrift von Bartley inkubiert, wobei der Kolben, der die Probe enthielt, bei 55°C und die Vorlage bei —10°C gehalten wurden. Nach 1 Std waren 92% des vorliegenden Acetats in das Destillat übergegangen.

b) Die Bestimmung des Acetats wurde nach der Vorschrift von Hepp et al. durchgeführt. Sie beruht auf der von Lundquist et al. vorgeschlagenen Acetylierung von Sulfonamid durch einen Enzymextrakt aus Taubenleber.

Enzymbestimmungen

Acetat-Thiokinase und Acetyl-CoA-Deacylase wurden im 100000 g-Überstand nach der Vorschrift von Hepp et al. bestimmt. Der Test für Acetat-Thiokinase beruht auf folgenden gekoppelten Reaktionen:

$$^{14}\text{C-Acetat} + \text{CoA} + \text{ATP} \xrightarrow{\text{Thiokinase}} {}^{14}\text{C-Acetyl-CoA} + \text{AMP} + \text{PP}$$

$$^{14}\text{C-Acetyl-CoA} + \text{Oxalacetat} \xrightarrow{\text{Citr. Synth.}} {}^{14}\text{C-Citrat} + \text{CoA}.$$

Das gebildete ^{14}C-Citrat wird als Silbersalz gefällt, isoliert und gezählt. Zeit- und Proteinabhängigkeitstests ergaben lineare Eichkurven.

Der Acetyl-CoA-Deacylasetest beruht auf der Entfärbung von 2:4 Dichlorphenol-Indophenol durch CoASH, das bei der enzymatischen Spaltung aus Acetyl-CoA freigesetzt wird.

Der Einbau von radioaktivem Acetat in Fettsäuren durch Extrakte aus Aortengewebe wurde nach der Methode von Numa et al. gemessen. Die Einzelheiten sind bei Hochheuser et al. beschrieben.

Ergebnisse und Diskussion

Plasmaacetatspiegel beim Miniaturschwein

a) Vergleich mit anderen Tierspecies (Tabelle 1). Das Miniaturschwein hat einen Plasmaacetatspiegel von 192 nMol/ml, ein Wert, der demjenigen des Hausschweins und der Ratte sehr nahe kommt. Jedoch liegt dieser Spiegel mindestens doppelt so hoch wie derjenige des Menschen. Eine Erklärung dafür könnte sein: Pflanzenfresser, wie z. B. die Ziege (Tabelle 1), haben einen außerordentlich hohen Blutacetatspiegel, was als Folge direkter Acetatabsorption aus dem Darm anzusehen ist. Es muß in Betracht gezogen werden, daß sowohl das Schwein als auch die Ratte, die unter Laboratoriumsbedingungen gehalten werden, eine beträchtliche Menge an Grünfutter mit der Nahrung zugeführt bekommen und daß dadurch der Blutacetatspiegel, entsprechend den Pflanzenfressern, ansteigen könnte.

Table 1

Tabelle 1. *Vergleich des Plasmaacetatspiegels in verschiedenen Tierspecies. Acetat wurde nach der Methode von* Hepp et al. *in heparinisiertem Plasma bestimmt. Vergleichswerte von anderen Tierspecies wurden aus der Arbeit von* Hochheuser et al. *sowie von* Lundquist et al. *entnommen*

Species	Anzahl der Untersuchungen	Plasmaacetat (nmol/ml)
Ratte	26	195 ± 10
Schwein (normal)	3	189 ± 6
Miniaturschwein	108	192 ± 4
Mensch	5	73 ± 20
Mensch (Lundquist)		50—100
Ziege (Menahan)		1000—1200

b) Einfluß der Ernährung und der Lebensbedingungen auf den Plasmaacetatspiegel (Tabelle 2). Zwei Punkte erscheinen hier von Interesse. Erstens die beträchtlichen Schwankungen des Acetatspiegels bei den verschiedenen Tiergruppen. Wir halten es nicht für wahrscheinlich, daß diese Schwankungen der Bestimmungsmethode zuzuschreiben sind. Vielmehr scheint es möglich zu sein, daß ein unterschiedlicher Streß bei der sehr eingreifenden Prozedur der Blutentnahme dafür verantwortlich sein könnte. Im Vergleich dazu sind die Werte von Weiss für die Ratte und von Lundquist für den Menschen bemerkenswert konstant. Bei diesen Versuchen wurde jeglicher Streß nach Möglichkeit ausgeschaltet. So wurden z. B. die Ratten unter besonders schonenden Bedingungen getötet (persönliche Mitteilung H. Weiss). Soweit die vorliegenden Daten ein

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Urteil zulassen, zeigten weder die Spezialfettdiät noch die unterschiedlichen Lebensbedingungen der Tiere einen auffallenden Effekt auf den Blutacetatspiegel. Vielleicht ist dieses Ergebnis nicht allzu überraschend, nachdem Ratten, die ausschließlich mit Speck gefüttert wurden, ebenfalls keine Änderung im Blutacetatspiegel aufwiesen (Weiss). Es wird

Table 2

Tabelle 2. *Plasmaacetatspiegel von Miniaturschweinen unter verschiedenen Bedingungen. Die Miniaturschweine (2 Monate alt zu Versuchsbeginn) waren in vier Gruppen zu je vier Tieren eingeteilt: Gruppe I und II erhielt eine 10%ige Spezialfettdiät*[1]*, Gruppe III und IV bekam Normalfutter. Gruppe I und III wurde im Stall gehalten, Gruppe II und IV im Freien. Plasmaacetat wurde im enteiweißten Plasma wie beschrieben bestimmt. Die Werte sind in nmol/ml angegeben; Mittelwerte ± SEM*

Date → Datum	Gruppe I	Gruppe II	Gruppe III	Gruppe IV
Oct. 20.10.1966	179 ± 45	139 ± 20	242 ± 62	78,8 (2)
Nov. 3.11.1966	64 ± 14	136 ± 28	146 ± 33	116 ± 18
22.11.1966	179 ± 35	220 ± 44	184 ± 33	183 ± 23
Jan. 11.1.1967	308 ± 51	146 ± 61	383 ± 39	287 ± 56
Mar. 7.3.1967	221 ± 49	148 ± 43	205 ± 33	239 ± 51
Apr. 24.4.1967	214 ± 15	179 ± 39	300 ± 39	280 ± 41
June 1.6.1967	75 ± 17	84 ± 8	88 ± 22	103 ± 3
Average values of group — Mittelwerte der Gruppe	176 ± 34	150 ± 19	221 ± 43	184 ± 30

[1] Die Fettsäurenzusammensetzung der Diät (Zöllner u. Tacconi): $C_{12:0}$ 0,3%; $C_{14:0}$ 1,6%; $C_{16:0}$ 55,6%; $C_{18:0}$ 25,8%; $C_{18:1}$ 14,0% und $C_{18:2}$ 2,7%.

Fatty acid composition of diet

deshalb von Interesse sein, den Blutacetatspiegel in einer fortgeschrittenen Phase der Fettfütterung zu verfolgen, d. h. bei älteren Tieren, bei denen es bereits zur Ausbildung arteriosklerotischer Gefäßveränderungen gekommen ist. Auch wird es von Vorteil sein, zur quantitativen Bestimmung des Acetats auf die exakter zu handhabende Methode der Gaschromatographie überzugehen.

Die Enzyme des Acetatstoffwechsels

Die Aktivität der Acetat-Thiokinase und der Acetyl-CoA-Deacylase in verschiedenen Geweben des Miniaturschweins sind in der Tabelle 3 aufgezeichnet. Eine Schätzung der potentiellen Acetatbildungsrate erhält man aus dem Quotienten Deacylase/Thiokinase; bei hohen Quotienten wird man ein Überwiegen der Acetatbildung erwarten (Hepp et al.). Wie aus Tabelle 3 ersichtlich, unterscheidet sich das Miniaturschwein in dieser Hinsicht nicht wesentlich von der Ratte. Die Aorta könnte eine Ausnahme bilden, obwohl die sehr niedrigen Enzymaktivitäten mit Vorsicht interpretiert werden müssen. Der hohe Quotient Deacylase/Thiokinase von 6,2 ließe an ein vorwiegend acetatproduzierendes Gewebe denken.

Dieser Punkt wurde noch nicht geprüft. Aus dem nächsten Experiment ist jedoch ersichtlich, daß trotz dieser schwachen Thiokinaseaktivität eine bemerkenswerte Fettsäuresynthese aus Acetat in Aortenrohextrakten möglich ist.

Table 3

Tabelle 3. *Thiokinase- und Deacylase-Aktivität in verschiedenen Organen des Miniaturschweins und der Ratte. Die Enzyme wurden nach den Methoden von* Hepp et al. *nach Aufschluß der tiefgefrorenen Proben in dem 100000 g-Überstand bestimmt. Das Alter der Miniaturschweine betrug 53 resp. 107 Tage. Die Werte sind in nmol/mg Protein/min angegeben (jeweils Mittelwerte ± SEM aus drei bis vier Bestimmungen, bei den Ratten aus vier bis sechs Bestimmungen)*

Organ	Miniaturschwein — Miniature swine			Ratte — Rat		
	Thiokinase	Deacylase	Deacylase/Thiokinase	Thiokinase	Deacylase	Deacylase/Thiokinase
Liver → Leber	2,63 ± 0,98	8,78 ± 2,87	3,35	5,57	9,77	1,7
Kidney → Niere	3,08 ± 1,22	5,89 ± 1,82	1,91	6,53	10,2	1,6
Heart → Herz	4,78 ± 1,59	12,1 ± 3,17	2,54	1,75	1,6	0,92
Aorta	0,32 ± 0,08	1,99 ± 0,9	6,2			

Fettsäuresynthese aus Acetat

Ein entscheidender Test für die Idee, daß die Höhe des Blutacetatspiegels und die Arteriosklerosenentstehung miteinander zusammenhängen, ist der Nachweis eines Fettsäure synthetisierenden Enzymsystems

Table 4

Tabelle 4. *^{14}C-Acetateinbau in Fettsäuren durch partikelfreien Aortenrohextrakt des Miniaturschweins und der Ratte. ^{14}C-Acetateinbau in Fettsäuren wurde nach der Methode von* Numa et al. *gemessen. Der partikelfreie Aortenrohextrakt wurde nach* Hochheuser et al. *hergestellt. Alle Werte sind in nmol/mg Protein/3 Std angegeben*

	^{14}C-Einbau in Fettsäuren (C14 incorporation into fatty acids)
Miniature swine — *Miniaturschwein*	
I	0,030
II	0,020
III	0,029
Rat — *Ratte* (Hochheuser) Mittelwerte von fünf Werten (Averages of 5 values)	0,035 ± 0,004

in dem entsprechenden Gewebe, nämlich der Aorta. Aus Tabelle 4 geht hervor, daß bei den drei untersuchten jungen Miniaturschweinen ziemlich gleichmäßige Einbauraten von ^{14}C-Acetat in Fettsäuren gefunden wurden. Die Tabelle 4 zeigt weiterhin, daß die Geschwindigkeit der Fettsäuresynthese in der Schweineaorta sich nicht wesentlich von derjenigen der Rattenaorta unterscheidet.

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Die Halbsättigungskonzentration für Acetat, d. h. die Acetatkonzentration, bei der die Fettsäuresynthese in vitro mit halbmaximaler Geschwindigkeit abläuft, wurde für die Rattenaorta zu „K_m" = $1{,}5 \times 10^{-4}$M bestimmt (Hochheuser et al.). Nachdem der Blutacetatspiegel der Ratte (Tabelle 1) $1{,}95 \times 10^{-4}$M beträgt, wäre es aus enzymkinetischen Überlegungen möglich, daß Schwankungen im Blutacetatspiegel die Geschwindigkeit der aortenständigen Fettsäuresynthese merklich beeinflussen könnten. In zukünftigen Versuchen soll deshalb auch die „K_m" in diesem System für die Aorta des Miniaturschweins bestimmt werden.

Mit Unterstützung durch die Deutsche Forschungsgemeinschaft.

Literatur

Bartley, W.: Biochem. J. **53**, 87 (1953).

Hepp, D., E. Freusse, H. Weiss u. O. Wieland: Biochem. Z. **344**, 87 (1966).

Hochheuser, W., H. Weiss u. O. Wieland: Z. klin. Chem. **2**, 175 (1964).

Kalich, J., K. Krumbacher u. B. Ruf: Z. ges. exp. Med. **145**, 312 (1968).

Lundquist, F., U. Fugmann, E. Klaning, and H. Rasmussen: Biochem. J. **72**, 409 (1959).

Menahan, L.: Persönliche Mitteilung.

Numa, S., M. Matsuhashi u. F. Lynen: Biochem. Z. **334**, 203 (1961).

Weiss, H.: Dissertation, München 1967.

Zöllner, N., and M. Tacconi: Nutr. et Dieta (Basel) **10**, 777 (1968).

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Comparative Studies of the Effect of Sodium Lactate, Sodium Acetate and Sodium Malate During Metabolic Acidosis

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1. Introduction

An acute metabolic acidosis, occurring, for example, as the result of circulatory arrest, can be quickly corrected (stopped) with sodium bicarbonate or THAM (1-4). In conditions with a persistent acidosis trend or as an addition to infusion solutions, alkalizing substances with a protracted (long-lasting) effect are necessary. Since sodium lactate, acetate and malate must first be metabolized in order to bind H ions, these compounds are used in different combinations as alkalizing adjuvants (additives) in infusion solutions. To test the therapeutic effectiveness of these compounds, animal experiments were carried out under medium-deep halothane narcosis and with a metabolic acidosis induced by ether narcosis.

2. Method

Tests were carried out on 20 mongrel dogs weighing between 18 and 33 kg; 30 min. after a morphine-atropine premedication (40 mg morphine and 0.5 mg atropine), the dogs were subjected to a narcosis with 8-12 mg/kg pento-barbital (Nembutal, supplied by Abbott, Frankfurt/Main). After intubation, the dogs were subjected to a controlled artificial respiration

from an Engström breathing apparatus. Respiration was regulated in such a way that the end CO_2 concentration in exhaled air, continuously measured through an ultra-red absorption spectrometer (Uras-M, from the Hartmann & Braun firm, Frankfurt/Main), was between 4.5 and 5.0 vol.%.

Na lactate (n=7), Na acetate (n=7) and Na malate (n=6) was infused into each dog twice in a single dose of 6.0 mval/kg. The test results obtained after the first infusion served as controls for the second test period (metabolic acidosis). The concentration of the solutions was in all cases equal to 400 mval/l. The infusion of the total amount, 270-490 ml of test solution, was done in 15 min. The observation time after each infusion was 2 hours. During the control phase (2 hrs), narcosis was maintained with 0.7 vol.% halothane (supplied by Farbwerke Höchst A. G., Frankfurt/Main). This was followed by a 2-hour-long deep ether narcosis (15, 10 and 8 vol.% ether every 40 min), in order to induce a metabolic acidosis prior to the second infusion. Both inhalation anesthetics used, namely halothane and ether, were vaporized with vapor instruments (halothane vapor and ether vapor of the Drägerwerke, Lübeck).

To measure the arterial and central venous pressure, pressure catheters were inserted through lateral branches of the brachial artery and vein into the ascending aorta and the vena cava. The three ECG (electrocardiogram) standard leads, the arterial and venous blood pressure, and the CO_2 exhaled were continuously recorded in an 8-channel recorder.

Prior to each infusion of the 3 test compounds and during the subsequent observation period (measurement intervals: 15-60 min), the following parameters were measured: pH, P_{CO_2}, standard bicarbonate, base excess (BE), hematocrit, serum electrolytes, lactate-pyruvate ratio in arterial whole blood, and oxygen absorption of the entire animal. All blood samples taken were collected with heparinized all-glass syringes. The pH, P_{CO_2},

standard bicarbonate and base excess values were determined by the Astrup method (5-7), and the required temperature corrections were made according to Severinghaus (8). The temperature was continuously measured with a rectally inserted mercury thermometer. Serum electrolytes were determined by flame photometry (Eppendorf flame photometer), and the lactate-pyruvate ratio was measured enzymatically (Biochemica Boehringer). Prior to the first control withdrawal and at the end of the test, the arterial oxygen saturation was checked (AO-photometer from the Hellige firm). A total of 5-7 ml blood was required for each blood withdrawal. The oxygen absorption was measured with the EHN spirometer (9). All values obtained were converted to 37°C body temperature (Q_{10}: 1.7), STPD (10), and ml/kg min.

Measurement of the amount of urine dischared was done with a catheter inserted into the bladder. In addition, during the entire duration of the test, 1000 ml of 10% glucose with 50 g levulose added was infused at a constant rate.

3. Results

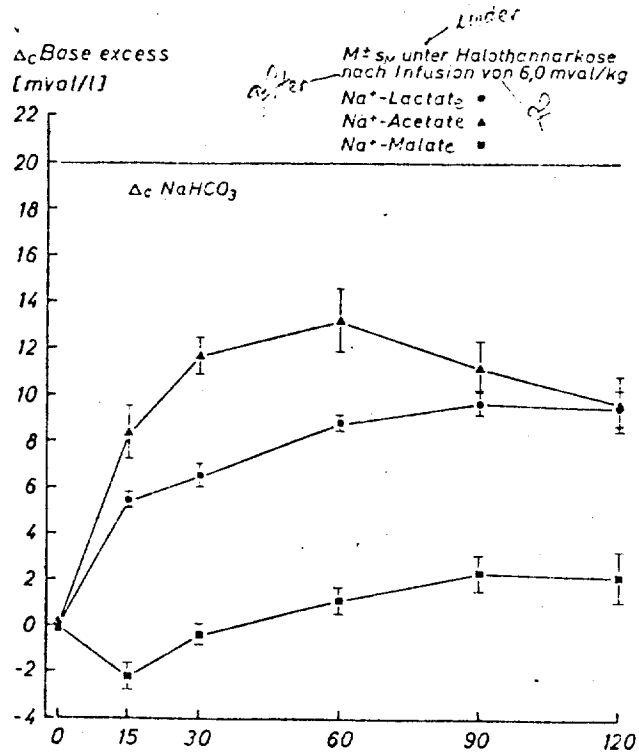
During the entire duration of the tests, the effects of respiration on the acid-base metabolism (balance) were excluded by controlled artificial respiration. Values for arterial P_{CO_2} and arterial oxygen saturation were within the normal range. The changes observed in the arterial pH, standard bicarbonate and base excess were of like orientation and thus an expression of purely metabolic changes.

In order to compare the effect of the 3 tested compounds, the change in base excess (ΔcBE[mval/l]) versus the value prior to the beginning of each infusion was selected as a measure.

Average values of ΔcBE after 6.0 mval/kg Na lactate, acetate and malate for the first test period are shown in Fig. 1. Already 30 min after the start of infusion, Na acetate causes an average change of BE of +11.7 mval/l; after 60 min, an alkalization peak of +13.2 mval/l is already reached, and after 2 hours a value of ±9.7 mval/l is still observed.

In contrast to the acetate, alkalization sets in at a slower rate after 6.0 mval/l Na lactate. A peak is reached only after 90 min with a ΔcBE of +9.7 mval/l; after 2 hours, no significant drop can be observed at +9.5 mval/l.

Fig. 1. Changes of base excess (ΔcBE) as compared to control values (halothane narcosis - first test period) after infusion of 6.0 mval/l Na lactate, acetate and malate.



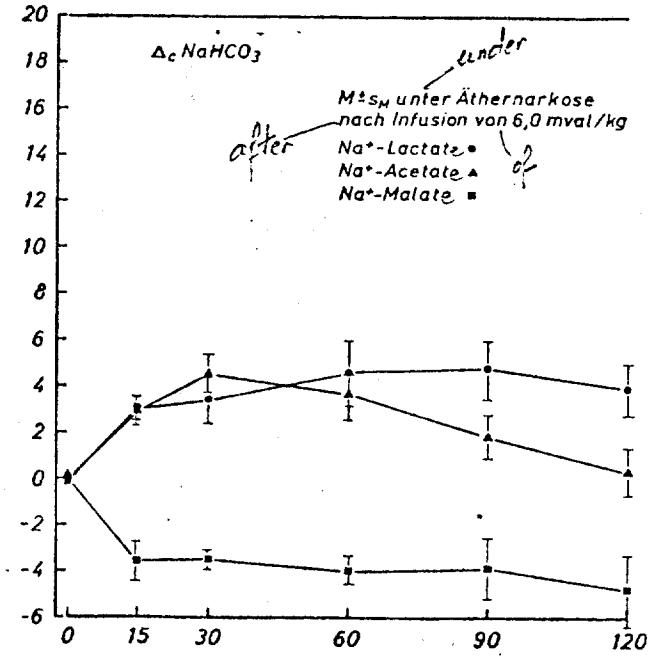
The effect of Na malate is completely different from that of Na acetate and lactate. After an initial acidification, a very slight alkalization sets in with a peak of ΔcBE of +2.2 mval/l after 2 hours.

A dose of 6.0 mval/kg of Na bicarbonate should yield a ΔcBE of +20 mval/l at a theoretical distribution volume of 30% of total body water. This value could be experimentally confirmed in a further test series (11), and is not reached by any of the 3 compounds tested here. The final value for lactate and acetate after 2 hours is half that great at +10 mval/l.

A 2-hour deep ether narcosis, following the control period, led to an average BE drop of 10 mval/l. Since the metabolic acidosis during the second test period was repressed by the ether narcosis, lower values for ΔcBE are found (Fig. 2). After Na acetate, a peak of the ΔcBE at +4.5 mval/l is already reached 30 min after the start of infusion. Then, a continuous drop of BE is observed, until the initial values are again reached after 2 hours. Na lactate leads, as in the first test period, to a protracted (delayed) alkalization. A peak of the ΔcBE at +4.6 mval/l is reached after about 60 min; after 2 hours, the effect only is slightly weaker with a ΔcBE of +3.9 mval/l. No alkalization is observed under ether after Na malate. The metabolic acidosis increases over the entire test period; 2 hours after the start of infusion, an average negative ΔcBE of 4.8 mval/l is noted.

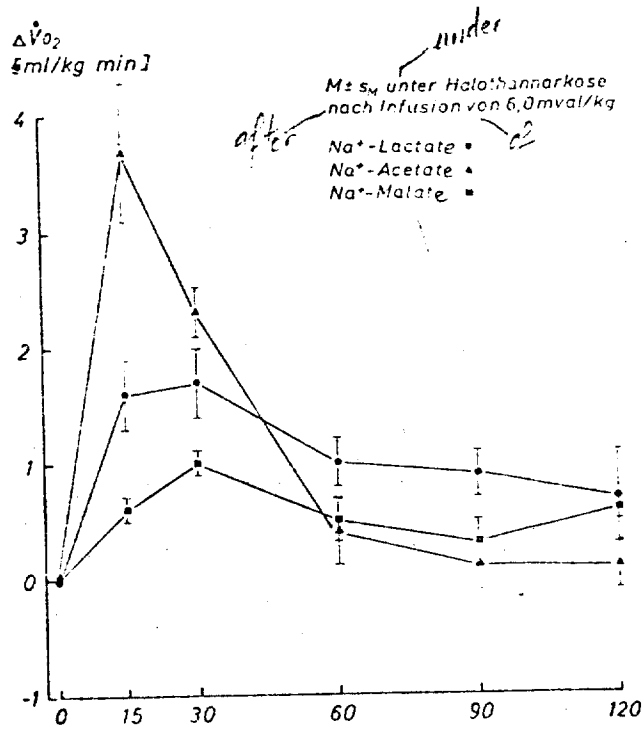
Fig. 2. Changes of base excess (ΔcBE) as compared to control values (ether narcosis - second test period) after infusion of 6.0 mval/kg Na lactate, acetate and malate.

Fig. 2.



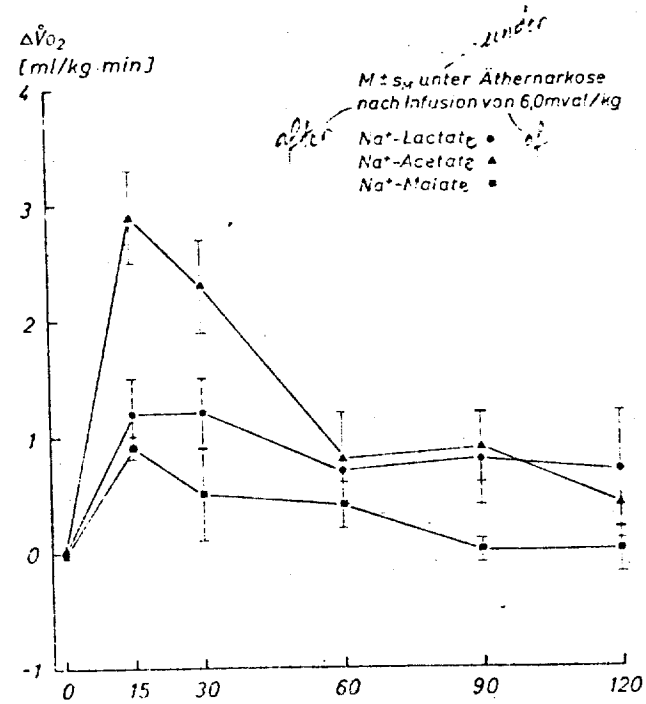
Changes of oxygen consumption ($\Delta V\text{-}O_2$) versus the control value for the first test period are shown in Fig 3. A striking factor is the great increase in oxygen consumption after Na acetate. Immediately after the end of infusion, an average $\Delta V\text{-}O_2$ of +3.7 ml/kg min and after a further 15 min of still +2.3 ml/kg min was measured; initial values are again reached 120 min after the beginning of infusion. After Na lactate, the $\Delta V\text{-}O_2$ peak at 1.6 ml/kg min is considerably lower. The increase in oxygen consumption continues up to the 30-min value, and then drops slowly; 2 hours after the start of infusion, slightly higher values of +0.7 ml/kg min are still observed. Infusion of Na malate causes only a slight increase in oxygen absorption, with a peak at +1.0 ml/kg min observed 30 minutes after the start of infusion.

Fig. 3. Changes in the oxygen absorption ($\Delta V\text{-}O_2$ (ml/kg min)) as compared to control values (halothane narcosis - first test period) after infusion of 6.0 mval/kg Na acetate, lactate and malate



As compared to halothane narcosis, a medium increase (50%) in oxygen absorption was noted under ether. In the second control period (metabolic acidosis), similar changes in oxygen absorption were noted for the 3 compounds tested. Fig. 4. shows the changes in oxygen consumption ($\Delta V\text{-}O_2$) as compared to the initial value for the second test period. Again, the great increase in oxygen consumption after Na acetate can be noted. Immediately after the end of infusion, an average $\Delta V\text{-}O_2$ of +2.9 ml/kg min and after another 15 min still of +2.5 ml/kg min was measured. The effect of Na lactate and malate on oxygen absorption is the same as in the first test period.

Fig. 4. Changes in oxygen absorption ($\Delta V\text{-}O_2$ (ml/kg min)) as compared to control values (ether narcosis-second test period) after infusion of 6.0 mval/kg. Na lactate, acetate and malate.



The considerable increase in oxygen absorption after Na acetate can be explained only in small part by the simultaneously observed tachycardia. In Table 1, the changes of the pulse rate (1/min) and of the venous pressure, as compared to control values at the end of infusion, are listed for the 3 compounds tested. Whereas a pulse rate increase of +23 and +14/min takes place after Na lactate and malate, a considerable tachycardia is noted after Na acetate, which returns to normal only 60 min after the beginning of infusion; when 6.0 mval/kg Na acetate and lactate are infused in 15 min at a rate of 400 mval/1, an average increase of the central venous pressure of 2.9 and 2.5 mm Hg, respectively, is noted. Infusion of Na malate produces a significantly greater (+4.1 mm Hg) increase of venous pressure ($p<0.0125$ and $p<0.005$, resp.).

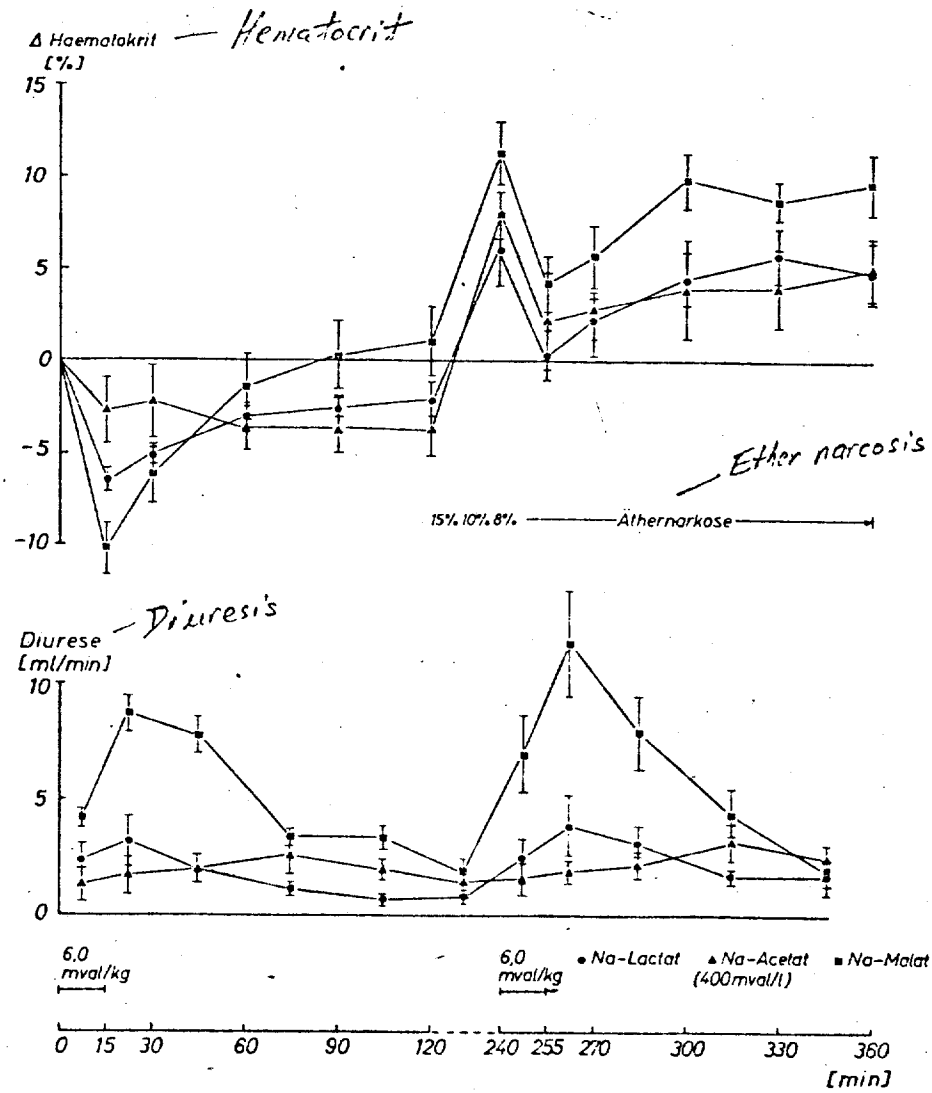
Table 1. Changes of the pulse rate (1/min) and of the venous pressure (mm Hg) after infusion of 6.0 mval/kg Na lactate, acetate and malate (400 mval/1 in 15 min.

	Δ Pulsfrequenz [1/min]			Δ $P_{ven.}$ [mm Hg]		
	Acetate	Lactate	Malate	Acetate	Lactate	Malate
pulse rate	+ 60	+22	−22	+3,0	+1,5	+6,5
	+ 72	± 0	−16	+5,6	+4,0	+7,0
	+144	+34	+56	+2,0	+0,5	+5,0
	+ 42	+22	+24	+3,0	+4,0	+3,0
	+ 72	− 8	−56	+4,0	±0,0	+4,5
	+ 18		+30	+4,5		+3,0
	+ 90	+94	+ 2	+0,5	+2,5	+3,5
	+ 50	+22	+ 8	+3,5	+2,5	+5,0
	+ 84	+32	+32	+1,0	+3,5	+4,5
	+ 38	+18	+32	+2,5	+2,5	+2,0
	+104	+24	−28	+1,5	+2,0	+3,5
	+ 38	+ 2	− 2	+3,0	+3,0	+2,0
	+ 14	+30		−4,0	+4,0	
	+ 9	+12		+3,0	+3,0	
M	+ 66,8	+23,4	+14,3	+2,9	+2,5	+4,1
n	14	13	12	14	13	12
s_M	12,4	6,9	8,2	0,4	0,4	0,5

As a result of the infusion of the test compounds, a hemodilution (hydremia) was observed in each case, which was most marked (highly expressed) after Na malate (Fig. 5.). During the second half of the tests, an increase of hematocrit was noted, which is particularly marked in the malate tests.

Fig. 5. Changes in hematocrit (ΔHkt) as compared to the control value of the first test period, and urine discharge (excretion) (ml/min) after a 2-time infusion of 6.0 mval/kg Na lactate, acetate and malate ($M \pm S_M$).

Fig. 5.



After Na lactate and Na acetate, no substrate-specific effect on the urine discarge could be observed (Fig. 5). In contrast to these 2 compounds, Na malate led to an excessive diuresis. The average urine volumes during the entire test period amounted to 244 ml for Na acetate and lactate, and 670 ml for Na malate.

In all 3 test series, serum electolytes exhibited the following changes: infusion of the compounds resulted in an immediate moderate increase of the serum sodium content and, parallel to blood dilution, in a drop of the serum potassium, calcium and chloride content.

Whereas the Na, Ca and Cl values again became normal within each test period, a continuous slight decrease of the serum potassium content was noted over the entire test period. Changes of the serum potassium after Na acetate were quite marked (1.8 mval/l), but after Na lactate and malate losses of 1.2 and 0.6 mval/l were observed. In the first test period, the lactate/pyruvate ratio did not exhibit any significant changes, as compared to controls, 60 and 120 min after infusion of Na acetate, lactate and malate. Under ether, a considerably greater lactate/pyruvate ratio was observed (12); however, 60 and 120 min after infusion no changes in this ratio were noted which could be interpreted as an effect of previous infusions of Na lactate, acetate and malate.

One animal (in the lactate test) died from a sudden ventricular fibrillation during the deep ether narcosis following the first test period. One dog (in the acetate test) died 2 hours after the end of the test from sudden hypoxia following extubation. After Na malate, the first 3 test animals were killed 18 hours after the end of the test in view of their extremely poor general condition. The cause of these changes could be explained as being due to the considerable metabolic acidosis (average BE: 11.1 mval/l, maximum values 17.4 mval/l),which was not corrected at the end of the test, and also to hemoconcentration (Fig. 5). For this reason, in the following tests, the acidosis, hemoconcentration and electrolyte deficiency were corrected at the end of the test by corresponding infusions. After this measure, all subsequent malate animals survived the 2-day limit set for the tests.

4. Discussion

Comparative studies of the effect of Na lactate, acetate and malate during undisturbed acid-base equilibrium and metabolic acidosis have not

been carried so far. The first 2 compounds, and also malate, are frequently components of infusion solutions. Their use provides, on the one hand, a parenteral, anion-free supply of different cations, and, on the other hand, infusion solutions exert a protracted (long-lasting) alkalizing effect as a result of the conversion of the lactate, acetate and malate anion to bicarbonate

In this study, concentrations of the test compounds higher than those found in commercially used infusion solutions were selected, in order that the effects on the acid-base equilibrium could be better observed from a quantitative standpoint. The hemodilution produced by infusion of the test compounds remained within representative limits and was predominantly balanced (adjusted, equalized) between 30 and 60 minutes after the beginning of infusion (Fig. 5.). The hemoconcentration observed during ether narcosis must be attributed to a discharge (voiding) of blood storage organs of the test animal (dog) after catecholamine liberation caused by ether (13).

Since in each test, during the first test period, the influence of an acid-base equilibrium undisturbed by anesthesia could be observed, and since it was shown in the same work group (14) that the metabolic acidosis produced by ether does not recede spontaneously during anesthesia, control tests were not considered necessary.

5. Conclusion

The results of this study show that a metabolic acidosis cannot be corrected by Na malate. The alkalizing effect of Na acetate is approximately the same as that of Na lactate, but sets in more rapidly and subsides (wears off) earlier. A rapid infusion of Na acetate leads to a marked stress of the circulation system, as a result of tachycardia and increased oxygen absorption. Na lactate also exerts a sufficient alkalizing effect in the

case of a considerable metabolic acidosis; the maximum effect is observed 60-90 min after the start of infusion and remains almost constant for over 2 hours. In view of this fact and its low stress on the circulation system, Na lactate seems to be the most suitable, of the 3 compounds tested, alkalizing component of infusion solutions.

For the treatment of an acute and severe metabolic acidosis, which must be rapidly relieved (corrected), sodium bicarbonate or THAM should preferably be used, rather than Na lactate and acetate.

Summary

Summary. Experiments with 20 mongrel dogs were made to study the influence of sodium acetate, -lactate and -malate (6 mval/kg) on acid-base equilibrium and on metabolic acidosis produced by deep ether anesthesia.

Following *sodium acetate* there was a remarkable increase of the base excess under normal conditions reaching its maximum value (+13.2 mval/l) 60 minutes after the application of the drug. Under ether anesthesia the alkalising effect has been considerably less, the peak of only +4.5 mval/l being gained already 30 minutes after the application. A fast infusion of sodium acetate was followed by an increased oxygen consumption (+3.7 ml/kg·min) and by tachycardia.

In comparison with these results the maximum rise of base excess following *sodium lactate* under normal conditions showed a delay of 30 minutes, the extent being slightly less (+9.7 mval/l) and lasting for a period of 2 hours. There was no significant difference between these two drugs under ether anesthesia concerning both duration and degree of alkalisation. The increase of oxygen consumption following sodium lactate gained its peak 30 minutes after the end of the infusion.

The effect of *sodium malate*, however, was quite different. In acid-base equilibrium an initial drop of base excess was followed by a merely slight trend to alkalisation, the maximum increase (+2.2 mval/l) being reached after two hours. During ether anesthesia this infusion did not produce any alkalising effect but distinctly intensified the metabolic acidosis. In contrast to the other two substances sodium malate furthermore led to an excessive diuresis. The data obtained suggest sodium lactate being the most suitable alkalising additive for non-colloidal infusions.

Bibliography

Literatur Tris

1. Nahas, G. G.: The pharmacology of TRIS (hydroxymethyl) amino-methane (THAM). Pharmacol. Rev. 14, 447 (1962).
2. Zimmermann, W. E.: Der Trispuffer in klinischer Anwendung. Dtsch. med. Wschr. 26, 1305 (1963).
3. — Anwendung und Wirkung von TRIS (hydroxymethyl) aminomethan (THAM). Pharm. Z. 19, 692 (1964).
4. Bleich, H. L., Schwartz, W. B.: Tris buffer (THAM) an appraisal of its physiologic effects and clinical usefulness. New Engl. J. Med. 274, 782 (1966).
5. Astrup, P.: A simple electrometric technique for the determination of carbon dioxide tension in blood and plasma, total content of carbon dioxide in plasma, and bicarbonate content in "separated" plasma at a fixed carbon dioxide tension (40 mm Hg). Scand. J. clin. Lab. Invest. 8, 33 (1956).
6. — Jorgensen, K., Siggaard-Andersen, O., Engel, K.: The acid-base metabolism, a new approach. Lancet 19601, 1035.
7. Siggaard-Andersen, O.: The pH-log P_{CO_2} blood acid-base nomogram revised. Scand. J. clin. Lab. Invest. 14, 598 (1962).
8. Severinghaus, J.W.: Blood gas calculator. J. Appl. Physiol. 21, 1108 (1966).
9. Engström, C.G., Herzog, P., Norlander, O.: A method for the continuous measurement of oxygen consumption in the presence of inert gases during controlled ventilation. Acta anaesth. scand. 5, 115 (1961).
10. Dokumenta Geigy, Wissenschaftliche Tabellen, Geigy AG, Basel, 1968.
11. Brückner, J. B.: Vergleichende Untersuchungen der Wirkung von Natrium-Bicarbonat und Tris (hydroxymethyl) aminomethan (THAM) bei metabolischer Azidose. Vortrag Anaesthesiekongr. Saarbrücken 1969 (im Druck).
12. Greene, N.M.: Lactate, pyruvate and excess lactate production in anesthetized man. Anesthesiology 22, 404 (1961).
13. Price, H.L.: Circulating adrenaline and noradrenaline during diethyl-ether anesthesia in man. Clin. Sci. 16, 377 (1957).
14. Rommelsheim, K.: Basen-Excess, arterieller Kohlensäuredruck und pH des Hundes unter Standardbedingungen und verschiedenen Narkosen. Inaug.-Diss. Köln 1968.

Vergleichende Untersuchungen der Wirkung von Natrium-Lactat, -Acetat und -Malat bei metabolischer Acidose

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1. Einleitung

Eine akut aufgetretene metabolische Acidose, z. B. als Folge eines Kreislaufstillstandes, läßt sich mit Natrium-Bicarbonat oder THAM rasch korrigieren [1—4]. Bei Zuständen mit einer anhaltenden Acidoseneigung oder als Zusatz von Infusionslösungen sind protrahiert wirkende alkalisierende Substanzen erwünscht. Da Natrium-Lactat, -Acetat und -Malat erst metabolisiert werden müssen, um H-Ionen binden zu können, werden diese Substanzen in verschiedenen Kombinationen als alkalisierende Adjuvantien von Infusionslösungen benutzt. Zur Prüfung des therapeutischen Wertes dieser Substanzen wurden Tierversuche in mitteltiefer Halothan-Narkose und bei einer durch Äthernarkose induzierten metabolischen Acidose vorgenommen.

2. Methodik

Die Untersuchungen wurden an 20 Bastardhunden im Gewicht zwischen 18,0 und 33,0 kg durchgeführt. 30 min nach einer Morphin-Atropin-Prämedikation (40,0 mg Morphin und 0,5 mg Atropin) wurde die Narkose mit Pentobarbital (Nembutal® der Firma Abbott, Frankfurt a. M.) — 8—12 mg/kg — eingeleitet. Nach Intubation erfolgte eine kontrollierte Beatmung mit reinem Sauerstoff durch einen Engström-Respirator. Die Atmung wurde so eingeregelt, daß die über ein Ultrarot-Absorptions-Spektrometer (Uras-M der Firma Hartmann & Braun, Frankfurt a. M.) kontinuierlich gemessene endexspiratorische CO_2-Konzentration zwischen 4,5 und 5,0 Vol-% lag.

Natrium-Lactat ($n = 7$), -Acetat ($n = 7$) und -Malat ($n = 6$) wurden jedem Hund zweimal in einer Einzeldosierung von 6,0 mval/kg infundiert. Die nach der ersten Infusion erhaltenen Untersuchungsergebnisse dienten als Kontrollen für die zweite Untersuchungsperiode (metabolische Acidose). Die Konzentration der Lösungen war in allen Fällen 400 mval/l. In 15 min erfolgte die Infusion der gesamten Menge — 270—490 ml-Testlösung. Der Beobachtungszeitraum betrug nach jeder Infusion 2 Std. Während der Kontrollphase (2 Std) wurde die Narkose durch 0,7 Vol.-% Halothan (Farbwerke Hoechst AG, Frankfurt a. M.) aufrechterhalten. Danach schloß sich eine zweistündige tiefe Äther-Narkose (je 40 min 15, 10 und 8 Vol.-% Äther) an, um vor der zweiten Infusion eine metabolische Acidose zu erzeugen. Während der zweiten Beobachtungsperiode wurde die Narkose durch 8 Vol.-% Äther in einem steady state gehalten. Die beiden verwendeten Inhalationsanaesthetica — Halothan und Äther — wurden mit Vapor-Geräten (Halothan-Vapor, Äther-Vapor der Drägerwerke, Lübeck) verdampft.

Zur Messung des arteriellen und zentral-venösen Drucks wurden Druckkatheter über Seitenäste der Arteria und Vena brachialis in die Aorta ascendens bzw. in die Vena cava vorgeschoben. Die drei EKG-Standard-Ableitungen, arterieller und venöser Blutdruck und das exspiratorische CO_2 wurden kontinuierlich auf einem 8-Kanal-Schreiber registriert.

Vor jeder Infusion der drei Testsubstanzen und während der folgenden Beobachtungsperiode (Meßintervalle 15—60 min) erfolgte die Messung folgender Parameter: pH, P_{CO_2}, Standardbicarbonat, Base-excess (BE), Hämatokrit, Serumelektrolyte, Lactat-Pyruvat-Quotient im arteriellen Vollblut und Sauerstoffaufnahme des Gesamttieres. Alle Blutentnahmen erfolgten in heparinisierte Glasspritzen. pH, P_{CO_2}, Standardbicarbonat und Base-excess wurden nach der Astrup-Methode ermittelt [5—7], erforderliche Temperaturkorrekturen erfolgten nach Severinghaus [8]. Die Temperatur wurde über ein rectal eingelegtes Quecksilberthermometer fortlaufend gemessen. Die Serumelektrolyte wurden flammenphotometrisch bestimmt (Flammenphotometer Eppendorf), der Lactat-Pyruvat-Quotient wurde enzymatisch gemessen (Biochemica Boehringer). Vor der ersten Kontrollentnahme und am Versuchsende erfolgte eine Kontrolle der arteriellen Sauerstoffsättigung (AO-Photometer der Firma Hellige). Für jede Blutentnahme wurden insgesamt 5—7 ml Blut benötigt. Die Messung der Sauerstoffaufnahme erfolgte mit dem EHN-Spirometer [9]. Alle dabei erhaltenen Werte wurden auf 37° C Körpertemperatur (Q_{10}: 1,7), STPD [10] und ml/kg·min umgerechnet.

Ein in die Harnblase eingelegter Katheter ermöglichte die Messung der Urinausscheidung.

Über die gesamte Versuchsdauer wurden zusätzlich 1000 ml 10%ige Glucose mit Zusatz von 50 g Lävulose gleichmäßig infundiert.

3. Ergebnisse

Während der gesamten Versuchsdauer waren durch die kontrollierte Beatmung respiratorische Einflüsse auf den Säure-Basen-Haushalt ausgeschaltet. Die Werte für den arteriellen P_{CO_2} und die arterielle Sauerstoffsättigung lagen im Normbereich. Die beobachteten Veränderungen des arteriellen pH, des Standardbicarbonates und des Base-excess waren gleichgerichtet und damit Ausdruck rein metabolischer Veränderungen.

Um die Wirkung der drei getesteten Substanzen vergleichen zu können, wurde als Maß die Änderung des Base-excess (ΔcBE [mval/l]) gegenüber dem Wert vor Beginn jeder Infusion gewählt. In Abb. 1 wurden die Mittelwerte des ΔcBE nach 6,0 mval/kg Na-Lactat, -Acetat und -Malat für die erste Versuchsperiode dargestellt. Na-Acetat führt schon 30 min nach Infusionsbeginn zu einer mittleren BE-Änderung von + 11,7 mval/l; nach 60 min ist bereits mit + 13,2 mval/l das Maximum der Alkalisierung erreicht; nach 2 Std findet sich noch ein Wert von + 9,7 mval/l.

Im Gegensatz zu Acetat setzt nach 6,0 mval/kg Na-Lactat die Alkalisierung verzögert ein. Das Maximum wird erst nach 90 min mit einem ΔcBE von

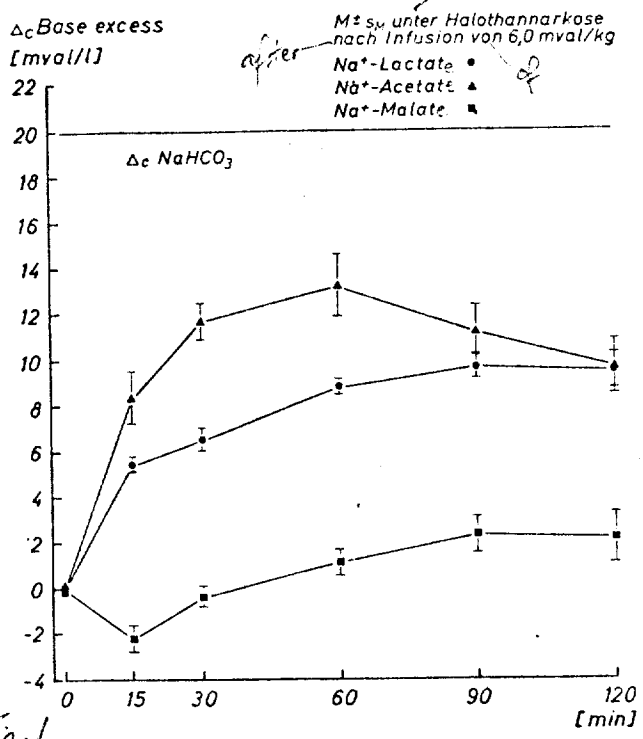

Abb. 1. Die Änderung des Base-excess (ΔcBE) gegenüber den Kontrollwerten (Halothannarkose — erste Versuchsperiode) nach Infusion von 6,0 mval/kg Natrium-Lactat, -Acetat und -Malat

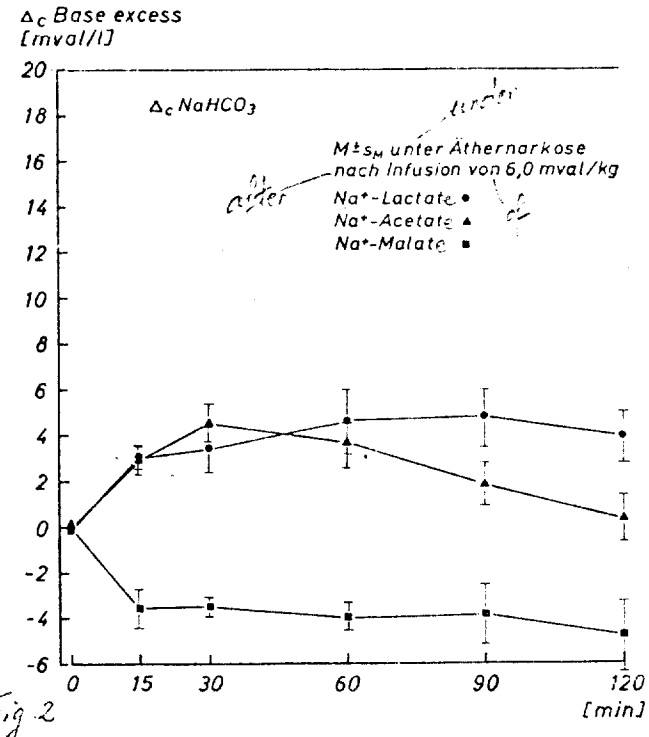

Abb. 2. Die Änderung des Base-excess (ΔcBE) gegenüber den Kontrollwerten (Äthernarkose — zweite Versuchsperiode) nach Infusion von 6,0 mval/kg Natrium-Lactat, -Acetat und -Malat

+9,7 mval/l erreicht. Nach 2 Std ist mit +9,5 mval/l noch kein wesentlicher Abfall zu beobachten.

Natrium-Malat zeigt gegenüber Na-Acetat und -Lactat eine völlig abweichende Wirkung: Nach einer initialen Säuerung setzt nur eine geringe Alkalisierung mit einem Maximum ΔcBE von +2,2 mval/l nach 2 Std ein.

Eine Dosis von 6,0 mval/kg Natrium-Bicarbonat sollte bei einem theoretischen Verteilungsvolumen von 30% des Gesamt-Körperwasser zu einem ΔcBE von +20 mval/l führen. Dieser Wert konnte in einer weiteren Untersuchungsserie experimentell bestätigt werden [11]. Er wird von keiner der drei hier getesteten Substanzen erreicht. Der Endwert für Lactat und Acetat nach 2 Std liegt etwa halb so hoch bei +10 mval/l.

Die zweistündige tiefe Äthernarkose im Anschluß an die Kontrollperiode führte zu einer mittleren BE-Senkung von 10 mval/l. Da während der zweiten Versuchsperiode die metabolische Acidose durch die Äthernarkose unterhalten wurde, finden sich niedrigere Werte für den ΔcBE (Abb. 2). Nach Natrium-Acetat wird das Maximum des ΔcBE schon 30 min nach Infusionsbeginn mit +4,5 mval/l erreicht. Danach ist ein kontinuierlicher Abfall des BE zu beobachten, bis nach 2 Std die Ausgangswerte wieder erreicht wurden. Na-Lactat führt ähnlich wie in der ersten Versuchsperiode zu einer protrahierten Alkalisierung. Das Maximum des ΔcBE ist nach etwa 60 min mit +4,6 mval/l erreicht; nach 2 Std ist die Wirkung mit einem ΔcBE von +3,9 mval/l erst gering abgeschwächt. Nach Natrium-Malat kommt es unter Äther zu keiner Alkalisierung. Die metabolische Acidose nimmt über die gesamte Untersuchungsperiode zu. Zwei Stunden nach Infusionsbeginn ist ein mittleres negatives ΔcBE von 4,8 mval/l erreicht.

In Abb. 3 wurde die Änderung des Sauerstoffverbrauchs ($\Delta\dot{V}$-O_2) gegenüber dem Kontrollwert für die erste Untersuchungsperiode dargestellt. Auffallend ist der starke Anstieg des Sauerstoffverbrauchs nach Na-Acetat. Unmittelbar nach Ende der Infusion wurde ein mittleres $\Delta\dot{V}$-O_2 von +3,7 ml/kg·min und nach weiteren 15 min wurden noch +2,3 ml/kg·min gemessen. 120 min nach Beginn der Infusion sind die Ausgangswerte wieder erreicht. Nach Na-Lactat liegt das Maximum des $\Delta\dot{V}$-O_2 mit +1,6 ml/kg·min wesentlich niedriger. Die Steigerung der Sauerstoffaufnahme hält noch bis zum 30 min-Wert an, um dann langsam abzufallen. Zwei Stunden nach Infusionsbeginn sind mit +0,7 ml/kg·min noch leicht erhöhte Werte zu beobachten. Die Infusion von Na-Malat bewirkt nur eine geringe Steigerung der Sauerstoffaufnahme mit einem Maximum von +1,0 ml/kg·min 30 min nach Infusionsbeginn.

Gegenüber der Halothan-Narkose kam es unter Äther zu einer mittleren Steigerung der Sauerstoffaufnahme von 50%. In der zweiten Kontrollperiode (metabolische Acidose) sind für die drei untersuchten Substanzen ähnliche Veränderungen der Sauerstoffaufnahme zu beobachten gewesen. In Abb. 4 wurde die Änderung des Sauerstoffverbrauchs ($\Delta\dot{V}$-O_2) gegenüber dem Ausgangswert für die zweite Untersuchungsperiode dargestellt. Wieder fällt der starke Anstieg des Sauerstoffverbrauchs nach Na-Acetat auf. Unmittelbar nach Ende der Infusion wurde ein mittleres $\Delta\dot{V}$-O_2 von +2,9 ml/kg·min und nach weiteren 15 min von noch +2,5 ml/kg·min gemessen. Natrium-Lactat und Natrium-Malat verhalten sich in ihrer Wirkung auf die Sauerstoffaufnahme ähnlich wie in der ersten Versuchsperiode.

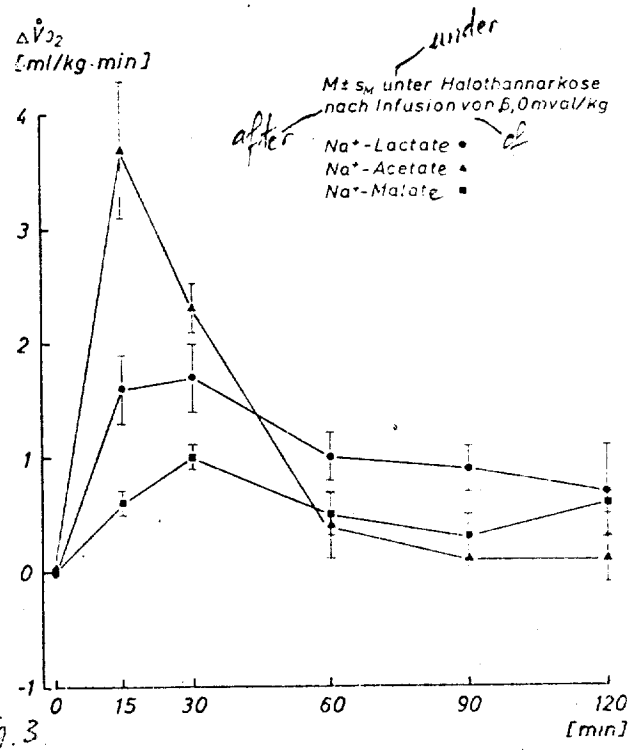

Fig. 3

Abb. 3. Die Änderung der Sauerstoffaufnahme ($\Delta \dot{V}O_2$ (ml/kg·min), gegenüber den Kontrollwerten (Halothannarkose — erste Versuchsperiode) nach Infusion von 6,0 mval/kg Natrium-Lactat, -Acetat und -Malat

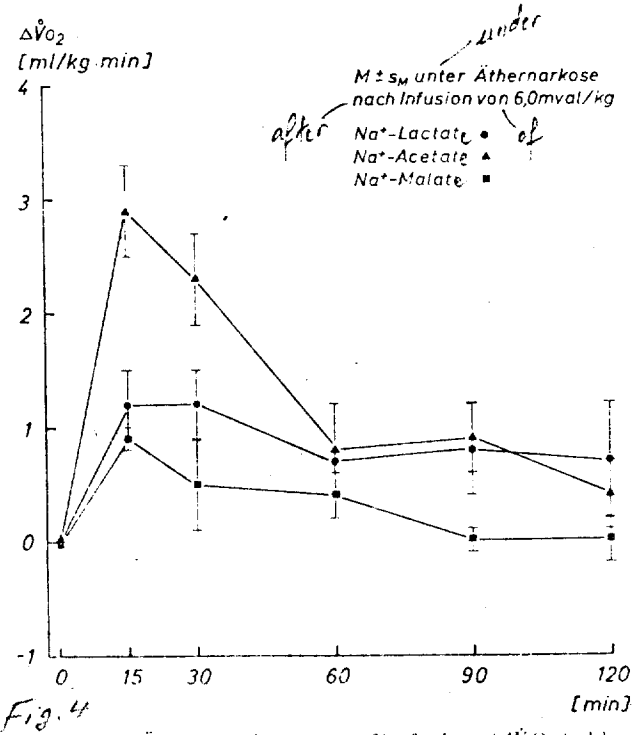

Fig. 4

Abb. 4. Die Änderung der Sauerstoffaufnahme ($\Delta \dot{V}O_2$ (ml/kg·min), gegenüber den Kontrollwerten (Äthernarkose — zweite Versuchsperiode) nach Infusion von 6,0 mval/kg Natrium-Lactat, -Acetat und -Malat

Tabelle 1.

Tabelle 1. *Die Veränderungen der Pulsfrequenz (1/min) und des venösen Druckes (mm Hg) nach Infusion von 6,0 mval/kg Natrium-Lactat, -Acetat und -Malat (400 mval/l) in 15 min*

	Δ Pulsfrequenz [1/min]			Δ $P_{ven.}$ [mm Hg]		
	Acetate	Lactate	Malate	Acetate	Lactate	Malate
	+ 60	+ 22	− 22	+ 3,0	+ 1,5	+ 6,5
	+ 72	± 0	− 16	+ 5,6	− 4,0	+ 7,0
	+ 144	+ 34	+ 56	+ 2,0	+ 0,5	+ 5,0
	− 42	− 22	+ 24	− 3,0	+ 4,0	+ 3,0
	− 72	− 8	− 56	+ 4,0	± 0,0	+ 4,5
	+ 18		+ 30	+ 4,5		+ 3,0
	+ 90	− 94	+ 2	+ 0,5	+ 2,5	− 3,5
	+ 50	− 22	+ 8	− 3,5	− 2,5	− 5,0
	− 84	+ 32	− 32	+ 1,0	+ 3,5	+ 4,5
	+ 38	+ 18	+ 32	+ 2,5	+ 2,5	+ 2,0
	+ 104	+ 24	− 28	+ 1,5	+ 2,0	− 3,5
	+ 38	+ 2	− 2	+ 3,0	+ 3,0	+ 2,0
	+ 14	− 30		+ 4,0	+ 4,0	
	− 9	− 12		+ 3,0	+ 3,0	
M	+ 66,8	+ 23,4	+ 14,3	+ 2,9	+ 2,5	+ 4,1
n	14	13	12	14	13	12
s_M	12,4	6,9	8,2	0,4	0,4	0,5

Die erhebliche Steigerung der Sauerstoffaufnahme nach Na-Acetat wird nur zum geringen Teil durch die gleichzeitig zu beobachtende Tachykardie erklärt. In Tabelle 1 wurden die Änderungen der Pulsfrequenz (1/min) und des Venendrucks gegenüber den Kontrollwerten nach Ende der Infusion für die drei getesteten Substanzen zusammengestellt. Während nach Na-Lactat und Na-Malat nur eine Pulsfrequenzzunahme von + 23 bzw. + 14/min eintritt, findet sich nach Acetat mit + 67/min eine erhebliche Tachykardie, die sich erst 60 min nach Infusionsbeginn normalisiert. 6,0 mval/kg von Na-Acetat und -Lactat (400 mval/l) in 15 min infundiert — führen zu einer mittleren Steigerung des zentral-venösen Drucks von 2,9 bzw. 2,5 mm Hg. Die Infusion von Na-Malat erzeugt mit + 4,1 mm Hg eine signifikant größere Venendrucksteigerung ($p < 0{,}0125$ bzw. $p < 0{,}005$).

Als Folge der Infusion der Testsubstanzen war jeweils eine Blutverdünnung (Abb. 5), die nach Malat am ausgeprägtesten ist, zu beobachten. Während der zweiten Hälfte der Versuche kommt es zu einem Anstieg des Hämatokrits, der bei den Malatversuchen besonders ausgeprägt ist.

Nach Na-Lactat und Na-Acetat ist keine substratspezifische Einwirkung auf die Urinausscheidung zu beobachten gewesen (Abb. 5). Natrium-Malat führt im Gegensatz zu diesen beiden Substanzen zu einer überschießenden Diurese. Die mittleren Harnvolumina lagen für die gesamte Versuchsdauer bei 244 ml (Acetat und Lactat) und 670 ml (Malat).

Die Serumelektrolyte zeigten bei allen drei Versuchsserien folgende typische Veränderungen: Die Infusion der Substanzen hat unmittelbar einen mäßigen Anstieg des Serum-Natriums und — parallel zur Blutverdünnung — einen Abfall des Serum-Kaliums, -Calciums und -Chlorids zur Folge. Während sich Na, Ca und Cl innerhalb jeder Untersuchungsperiode wieder normalisierten, kam es über die gesamte Versuchsdauer zu einer fortschreitenden leichten Verminderung des Serum-Kaliums. Die Veränderungen des Serum-Kaliums waren nach Acetat mit − 1,8 mval/l ausgeprägt, nach Lactat und Malat fanden sich Verluste von 1,2 bzw. 0,6 mval/l. Der Lactat-Pyruvat-Quotient wies in der ersten Versuchsperiode 60 und 120 min nach Infusion von Na-Lactat, -Acetat und

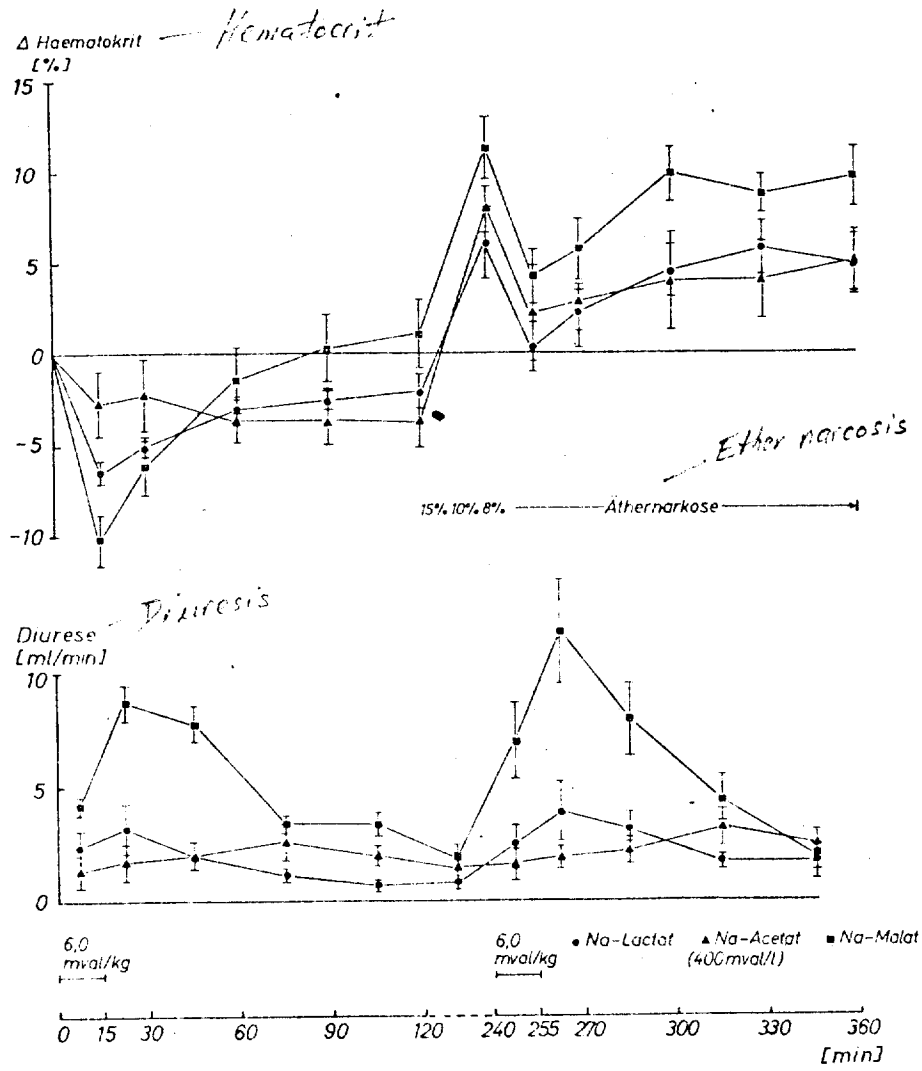

Fig. 5

Abb. 5. Die Veränderung des Hämatokrits (Hkt) im Vergleich zum Kontrollwert der ersten Untersuchungsperiode und die Urinausscheidung (ml/min) nach zweimaliger Infusion von 6,0 mval/kg Na-Lactat, -Acetat und -Malat ($M \pm s_M$)

-Malat keine wesentlichen Veränderungen, verglichen mit den Kontrollen, auf. Unter Äther war ein z.T. erheblich vergrößerter Lactat/Pyruvat-Quotient zu beobachten [12]. 60 und 120 min nach Infusion waren keine Veränderungen des Quotienten eingetreten, die als Einwirkungen der vorausgegangenen Infusionen von Lactat, Acetat und Malat hätten gedeutet werden können.

Ein Tier (Lactatversuch) starb durch plötzlich auftretendes Kammerflimmern während der auf die erste Untersuchungsperiode folgenden tiefen Äthernarkose. Ein Hund (Acetatversuch) verstarb 2 Std nach Versuchsende an einer plötzlichen Hypoxie, im Anschluß an die Extubation. Die ersten drei Versuchstiere nach Malat wurden 18 Std nach Versuchsende wegen extrem schlechten Allgemeinzustandes getötet. Die Ursache dieser Veränderungen konnte mit der bei Versuchsende nicht korrigierten erheblichen metabolischen Acidose (mittlerer BE −11,1 mval/l; Maximalwerte −17,4 mval/l) und Hämokonzentration erklärt werden (Abb. 5). Bei den folgenden Versuchen wurden deshalb Acidose, Hämokonzentration und Elektrolytdefizite nach Versuchsende durch entsprechende Infusionen korrigiert. Danach überlebten alle folgenden Malat-Tiere die gesetzte 2-Tages-Grenze.

4. Diskussion

Vergleichende Untersuchungen der Wirkung von Na-Lactat, -Acetat und -Malat bei nicht gestörtem Säure-Basen-Gleichgewicht und bei einer metabolischen Acidose liegen bisher nicht vor. Die ersten beiden Substanzen, aber auch Malat, sind häufiger Bestandteil von Infusionslösungen. Ihre Verwendung bezweckt einmal die parenterale anionenfreie Zufuhr verschiedener Kationen, zum anderen sollen die Infusionslösungen durch die Umwandlung des Lactat- bzw. Acetat- oder Malat-Anions in Bicarbonat einen protrahierten alkalisierenden Effekt entfalten.

In dieser Untersuchung wurden höhere Konzentrationen der Testsubstanzen, als in den kommerziell verwendeten Infusionslösungen zu finden sind, gewählt, um die Wirkungen auf das Säure-Basen-Gleichgewicht quantitativ besser beobachten zu können. Die durch die Infusion der Testsubstanzen erzeugte Hämodilution blieb in vertretbaren Grenzen und war zwischen 30 und 60 min nach Infusionsbeginn schon überwiegend ausgeglichen (Abb. 5). Die während der Äthernarkose beobachtete Hämokonzentration ist auf eine Entleerung der Blutspeicher des Versuchstieres Hund nach äthérbedingter Katecholaminausschüttung zurückzuführen [13].

Da in jedem Versuch während der ersten Untersuchungsperiode die Beeinflussung eines durch die Anaesthesie nicht gestörten Säure-Basen-Gleichgewichtes beobachtet werden konnte und in der gleichen Arbeitsgruppe [14] gezeigt wurde, daß die durch Äther erzeugte metabolische Acidose während der Anaesthesie sich nicht spontan zurückbildet, wurden Kontrollversuche nicht für notwendig erachtet.

5. *Schlußfolgerung*

Na-Malat ist nach den Ergebnissen dieser Untersuchung nicht geeignet, eine metabolische Acidose zu korrigieren. Die alkalisierende Wirkung bei Na-Acetat ist von annähernd gleicher Größe wie diejenige von Na-Lactat, setzt aber rascher ein und klingt früher ab. Na-Acetat führt bei rascher Infusion infolge einer Tachykardie und einer Steigerung der Sauerstoffaufnahme zu einer ausgeprägten Kreislaufbelastung. Na-Lactat wirkt auch bei einer erheblichen metabolischen Acidose ausreichend alkalisierend, die maximale Wirkung liegt 60—90 min nach Infusionsbeginn und hält über 2 Std nahezu gleichbleibend an. Dieser Umstand und die geringe Kreislaufbelastung lassen Na-Lactat von den drei untersuchten Substanzen als alkalisierenden Bestandteil von Infusionslösungen am besten geeignet erscheinen.

Für die Therapie einer akut aufgetretenen schweren metabolischen Acidose, die schnell korrigiert werden muß, sind Natrium-Bicarbonat oder THAM dem Lactat und Acetat vorzuziehen.

Summary. Experiments with 20 mongrel dogs were made to study the influence of sodium acetate, -lactate and -malate (6 mval/kg) on acid-base equilibrium and on metabolic acidosis produced by deep ether anesthesia.

Following *sodium acetate* there was a remarkable increase of the base excess under normal conditions reaching its maximum value (+13.2 mval/l) 60 minutes after the application of the drug. Under ether anesthesia the alkalising effect has been considerably less, the peak of only +4.5 mval/l being gained already 30 minutes after the application. A fast infusion of sodium acetate was followed by an increased oxygen consumption (+3.7 ml/kg·min) and by tachycardia.

In comparison with these results the maximum rise of base excess following *sodium lactate* under normal conditions showed a delay of 30 minutes, the extent being slightly less (+9.7 mval/l) and lasting for a period of 2 hours. There was no significant difference between these two drugs under ether anesthesia concerning both duration and degree of alkalisation. The increase of oxygen consumption following sodium lactate gained its peak 30 minutes after the end of the infusion.

The effect of *sodium malate*, however, was quite different. In acid-base equilibrium an initial drop of base excess was followed by a merely slight trend to alkalisation, the maximum increase (+2.2 mval/l) being reached after two hours. During ether anesthesia this infusion did not produce any alkalising effect but distinctly intensified the metabolic acidosis. In contrast to the other two substances sodium malate furthermore led to an excessive diuresis. The data obtained suggest sodium lactate being the most suitable alkalising additive for non-colloidal infusions.

Literatur

1. Nahas, G. G.: The pharmacology of TRIS (hydroxymethyl) amino-methane (THAM). Pharmacol. Rev. **14**, 447 (1962).
2. Zimmermann, W. E.: Der Trispuffer in klinischer Anwendung. Dtsch. med. Wschr. **26**, 1305 (1963).
3. — Anwendung und Wirkung von TRIS (hydroxymethyl) aminomethan (THAM). Pharm. Z. **19**, 692 (1964).
4. Bleich, H. L., Schwartz, W. B.: Tris buffer (THAM) an appraisal of its physiologic effects and clinical usefulness. New Engl. J. Med. **274**, 782 (1966).
5. Astrup, P.: A simple electrometric technique for the determination of carbon dioxide tension in blood and plasma, total content of carbon dioxide in plasma, and bicarbonate content in "separated" plasma at a fixed carbon dioxide tension (40 mm Hg). Scand. J. clin. Lab. Invest. **8**, 33 (1956).
6. — Jorgensen, K., Siggaard-Andersen, O., Engel, K.: The acid-base metabolism, a new approach. Lancet **19601**, 1035.
7. Siggaard-Andersen, O.: The pH-log P_{CO_2} blood acid-base nomogram revised. Scand. J. clin. Lab. Invest. **14**, 598 (1962).
8. Severinghaus, J. W.: Blood gas calculator. J. Appl. Physiol. **21**, 1108 (1966).
9. Engström, C. G., Herzog, P., Norlander, O.: A method for the continuous measurement of oxygen consumption in the presence of inert gases during controlled ventilation. Acta anaesth. scand. **5**, 115 (1961).
10. Dokumenta Geigy, Wissenschaftliche Tabellen, Geigy AG, Basel, 1968.
11. Brückner, J. B.: Vergleichende Untersuchungen der Wirkung von Natrium-Bicarbonat und Tris (hydroxymethyl) -aminomethan (THAM) bei metabolischer Azidose. Vortrag Anaesthesiekongr. Saarbrücken 1969 (im Druck).
12. Greene, N. M.: Lactate, pyruvate and excess lactate production in anesthetized man. Anesthesiology **22**, 404 (1961).
13. Price, H. L.: Circulating adrenaline and noradrenaline during diethyl-ether anesthesia in man. Clin. Sci. **16**, 377 (1957).
14. Rommelsheim, K.: Basen-Excess, arterieller Kohlensäuredruck und pH des Hundes unter Standardbedingungen und verschiedenen Narkosen. Inaug.-Diss. Köln 1968.

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THE BIOSYNTHESIS OF CHOLESTEROL FROM ACETATE-1-C^{14} BY CELLULAR FRACTIONS OF RAT LIVER*

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We have previously reported the finding that cell-free preparations of rat liver are capable of forming radioactive cholesterol from C^{14}-labeled acetate (1, 2). This has opened the way to an investigation of the possible contributions to the process made by the various structural elements of the cell. The mitochondria and microsomes have been segregated from the soluble cell components by differential centrifugation, and the ability of these fractions to carry out this synthesis has been tested singly and in combination. The cofactor and oxygen requirements, the effect of certain enzyme inhibitors, and the relation of the activity of the system to glycolysis have also been examined.

EXPERIMENTAL

Animals—Female albino rats, originally derived from Wistar stock and weighing approximately 200 gm., were sacrificed by decapitation.

Preparation of Homogenates—The livers were perfused *in situ* for a few seconds with ice-cold buffer medium containing 0.1 M potassium phosphate buffer, pH 7.4, 0.03 M nicotinamide, 0.004 M $MgCl_2$, and 0.125 M sucrose, and then homogenized as described previously (1, 2) in the same medium. The sucrose was introduced in the hope that it would reduce agglutination of cytoplasmic particles and thus expedite fractionation; its omission made little demonstrable difference in the activity of the preparation. In later experiments 0.01 M glutathione was added prior to homogenization because, in its presence, the activity of some preparations was increased.

Fractionation—Homogenates were centrifuged for 10 minutes at 500 or 700 × *g* to eliminate tissue fragments, cells, and nuclei. Removal of this material did not cause any loss of synthetic capacity. The resultant supernatant fluid was the starting material for subsequent fractionation, which

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was carried out in the high speed attachment of the refrigerated International centrifuge, model PR-2, and in the Spinco preparative ultracentrifuge when the force exceeded 30,000 × *g*. Details of the separate experiments are given below. The supernatant fluid was separated from the sediment after each centrifugation by careful aspiration with a finely drawn out pipette. The morphological composition of each fraction was determined by phase contrast microscopy.

The sediments, which contained mitochondria or microsomes, were resuspended in either buffer medium or supernatant fluid, as required, by homogenization for a few seconds in a glass tube with a loose fitting stainless steel or glass pestle in the case of the mitochondria, and a tight fitting plastic pestle in the case of the microsomes.

Incubation— In most instances incubation beakers contained 2 ml. of homogenate or particle suspension in buffer medium plus diphosphopyridine nucleotide (DPN) and acetate-1-C^{14} [1] and other additions as specified, in a final volume of 2.5 ml. All added substances were adjusted to approximately neutral pH with KOH, and all substrates converted to the potassium salts before use. Hexose diphosphate was first purified through the strychnine salt (3) to remove inhibitors which were found in all commercial preparations tested. Versene (ethylenediaminetetraacetic acid, disodium salt) was introduced in some instances because it increased the activity of the system, presumably by sequestration of metallic impurities, especially in the presence of certain added substances.

Beakers were incubated aerobically in a Dubnoff shaker in duplicate for 1 or 2 hours at 37°. The activity of the homogenates varied from one preparation to another, but results were consistent within experiments. Each experiment was carried out at least twice, and typical runs were tabulated.

Analyses— To determine the amount of labeled cholesterol, hydrolysis of the incubated sample, extraction with petroleum ether, precipitation, and counting of the cholesterol as the solid digitonide were carried out as described previously (2). Total cholesterol was determined by a modification of the Schoenheimer-Sperry method in the studies *in vitro* (4, 5) and by weight of the cholesterol digitonide in the experiments *in vivo*.

For measurement of the protein content of the incubation mixture, two methods were employed, one based on the weight of dry protein after extraction of nucleic acids and lipides (2), and in later experiments one based on biuret reaction (6). In the latter case normal liver protein of known dry weight was used as a standard of reference. Ribonucleic acid determinations were carried out by the method of Scott *et al.* (7).

[1] For synthesis of the labeled acetate, we are very grateful to Dr. Ivan D. Frantz, Jr., of the University of Minnesota School of Medicine.

Results

Experiments in Vitro: Fractionation and Recombination of Particulates and Soluble Cell Components— The results of two typical fractionation and recombination experiments are shown in Table I. The starting material was the supernatant fluid derived from centrifugation of homogenates for 10 minutes at 700 × *g*. This fluid was found to contain principally mitochondria, microsomes, lipide droplets, and soluble cell constituents; rarely, free nuclei were present, but never any whole cells.

TABLE I

Total Radioactivity Recovered in Cholesterol after Incubation of Various Liver Cell Fractions with Acetate-1-C^{14}

Tissue preparation	Homogenized and fractionated in	
	Buffer medium	0.25 M sucrose
	*c.p.m.**	*c.p.m.**
Unfractionated 700 × *g* supernatant	100	100
9,000 × *g* sediment	0	0
105,000 × *g* sediment	0	0
105,000 × *g* supernatant	0	0
9,000 × *g* + 105,000 × *g* sediments	0	0
9,000 × *g* sediment (washed) + 105,000 × *g* supernatant	19	25
105,000 × *g* sediment + 105,000 × *g* supernatant	82	70
9,000 + 105,000 × *g* sediments + 105,000 × *g* supernatant	102	95

* Counts per minute corrected to 100 in the unfractionated preparation to facilitate comparison between experiments. In the "Buffer medium" series, the particles were resuspended in either homogenizing medium or in soluble fraction (supernatant fluid from 105,000 × *g*) when its presence was required. Each incubation beaker contained 2 ml. of tissue fractions plus 0.0008 M DPN and 0.012 M acetate-1-C^{14} (8 × 10^4 c.p.m. per flask) in a total of 2.5 ml. In the "0.25 M sucrose" series the particles were resuspended in either 0.25 M sucrose or soluble fraction, and phosphate buffer, $MgCl_2$, DPN, and acetate were introduced to correspond to levels in the "Buffer medium" series. In addition, hexose diphosphate (0.016 M) and disodium Versenate (0.001 M) were present, bringing the final volume to 2.7 ml.

The large particles were sedimented by centrifugation for 15 minutes at 9000 × *g*. This fraction contained nearly all of the mitochondria from the unfractionated material, but also many microsomes. Centrifugation of the remaining supernatant fluid for 30 minutes at 105,000 × *g* yielded a microsomal pellet with characteristic reddish, translucent appearance and in which comparatively few particles were positively identifiable as mitochondria.

The final supernatant fluid contained the soluble constituents of the cell. Numerous lipide droplets accumulated in a layer at the top of the tube,

but there were otherwise few visible granules. No attempt was made to isolate this fatty layer, which was pooled with and treated as part of the soluble fraction.

Because of contamination with microsomes, the mitochondrial fraction was resuspended and resedimented twice at 5000 × *g*. In one set of experiments (Table I, "Buffer medium"), the soluble fraction, derived from centrifugation at 105,000 × *g*, was used for this washing, instead of the usual homogenizing medium, in order to minimize the possible elution of essential components from the mitochondria. In a second set of experiments (Table I, "0.25 M sucrose"), to avoid adding electrolytes which are reported to promote agglutination of particles (8-10), the entire homogenization, fractionation, and washing procedure were carried out in 0.25 M sucrose solution, buffer and salts being added in concentrated form at the time of incubation. Since the total amount of C^{14} incorporation tended to be lower in the material prepared in sucrose, hexose diphosphate and Versene were also introduced in order to boost activity.

Both the mitochondrial and microsomal pellets contained appreciable amounts of glycogen which could be seen grossly as a separate, slightly milky, translucent layer at the bottom of the centrifuge tubes. In the sediment from centrifugation at 105,000 × *g* the glycogen layer was at least equal in volume to the microsomal layer.

For incubation, the particles were resuspended in enough homogenization medium or soluble fraction to restore their concentration to approximately that in the original starting material; *i.e.*, supernatant fluid obtained from centrifugation at 700 × *g*.

Fractionation in buffer medium and in sucrose solution yielded essentially the same results (Table I). Although all of the fractions were ineffective by themselves, the activity of the system could be completely restored if they were recombined in their original proportions. Further, each one of the particulate fractions, when combined with the soluble portion, was capable of forming radioactive cholesterol from labeled acetate, but the microsomes were far more active in this regard than the washed mitochondria. Since the crude mitochondrial fraction was heavily contaminated with microsomes, and since washing these mitochondria was found to reduce their activity by about 75 per cent, the microsomes seemed the more likely site of the cholesterol-forming enzymes. No activity was ever obtained by incubation of the soluble fraction alone, even when it was fortified with hexose diphosphate to compensate for the glycogen lost by high speed centrifugation (see below).

To define more clearly the nature of the particles involved, sediments were collected by successive centrifugations as follows: (1) 10 minutes at 700 × *g*, (2) 15 minutes at 30,000 × *g*, (3) 15 minutes at 60,000 × *g*, and

(4) 90 minutes at 105,000 × *g*. Most of the mitochondria were in the first sediment, but a number of microsomes were also clearly visible. The mitochondria were not washed in this experiment. The remaining microsomes were arbitrarily subdivided into three fractions: the first, collected at 30,000 × *g*, contained principally large microsomes, although some mitochondria were seen. The other two, collected at higher speeds, contained

TABLE II

Cholesterol Formation by Various Classes of Cell Particles Incubated with Constant Amount of Soluble Fraction

Tissue preparation	Volume of particles added	Particle protein per beaker	RNA per beaker	Cholesterol per beaker
	ml.	*mg.*	*mg.*	*c.p.m.*
Unfractionated 700 × *g* supernatant	(2.0)*	(60.0)*	2.12	1127
5,000 × *g* sediment	*0.5*†	*15.9*	*1.07*	*279*
5,000 × *g* "	0.2	6.4	0.642	92
30,000 × *g* sediment	*0.5*	*10.7*	*0.966*	*825*
30,000 × *g* "	0.2	4.3	0.600	283
30,000 × *g* "	0.1	2.1	0.478	121
60,000 × *g* "	*0.5*	*8.3*	*1.22*	*417*
60,000 × *g* "	0.2	3.3	0.700	154
60,000 × *g* "	0.1	1.7	0.528	57
105,000 × *g* "	*0.5*	*6.8*	*1.91*	*25*
105,000 × *g* "	0.2	2.7	0.690	5

* The tissue preparation (in parentheses) in these beakers consisted only of unfractionated supernatant fluid. Hence the protein value includes the amount present in particulates plus the soluble fraction. Particles were collected by centrifugation at the forces indicated, and resuspended in a small volume of buffer medium. Aliquots of 0.5, 0.2, or 0.1 ml. of suspended particles were incubated with 2.0 ml. of soluble fraction. The latter contained 41.3 mg. of protein and 0.356 mg. of RNA. Addition of 0.0008 M DPN, 0.012 M acetate-1-C^{14}, 0.008 M hexose diphosphate, 0.001 M disodium Versenate, and medium as required brought the final volume to 2.76 ml.

† To facilitate comparisons, the preparations containing the highest concentration of particles in each class are italicized.

only microsomes. Aliquots of suspensions of each class of particles were incubated with a constant amount of soluble fraction as shown in the experiment in Table II; in this way, the activities of the various classes of particles could be compared at equivalent levels of particle protein concentration. The incorporation increased with particle concentration within each class of particles. The two large microsomal fractions were far more active than the others. This observation is even more striking when considered in terms of incorporation relative to the amount of particle protein present; the upper part of Fig. 1 shows the results of this and another such

experiment, expressed as counts per minute per mg. of particle protein corrected to 100 c.p.m. in the most active fraction, to facilitate comparison between experiments. The results of the two experiments were in excel-

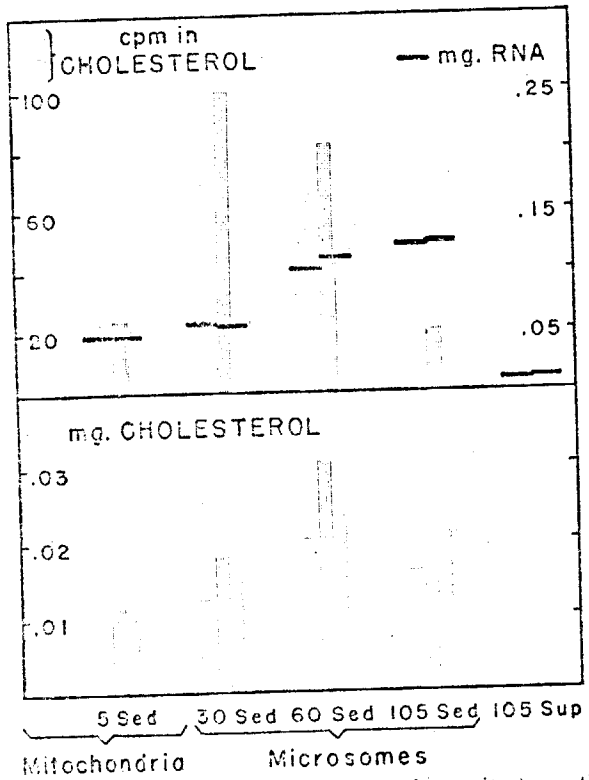

FIG. 1. Cholesterol and RNA in cell fractions, and capacity to synthesize cholesterol *in vitro*. The vertical bars in the upper part show, in two separate experiments, the relative capacities of the various cellular fractions to synthesize cholesterol. The data are expressed as counts per minute incorporated into cholesterol per mg. of particle protein, corrected to 100 c.p.m. in the highest fraction. The black horizontal lines show the concentration of RNA in the cell particles, expressed as mg. of RNA per mg. of particle protein (scale at right). In the lower part, the vertical bars show the cholesterol concentration expressed as mg. of cholesterol per mg. of particle protein in the various cell fractions in three separate experiments.

lent agreement and demonstrate clearly that the major activity resided in the sediments collected at 30,000 and 60,000 × *g*.

The lower part of Fig. 1 shows that the cholesterol concentration per mg. of particle protein was greatest in the three microsomal fractions, and least in the soluble portion. It approximately paralleled the synthetic

capacity of the particles, although it was moderately high in the smallest microsomes (sediment from 105,000 × *g*) in which the activity was very low. The appreciable amount of cholesterol in the mitochondrial sediment (5000 × *g*) was probably attributable to microsomal contaminants (see below).

There appeared to be no correspondence between the ribonucleic acid (RNA) content of the particles and their ability to form cholesterol (Table II and Fig. 1). The smallest particles, which were richest in RNA, were

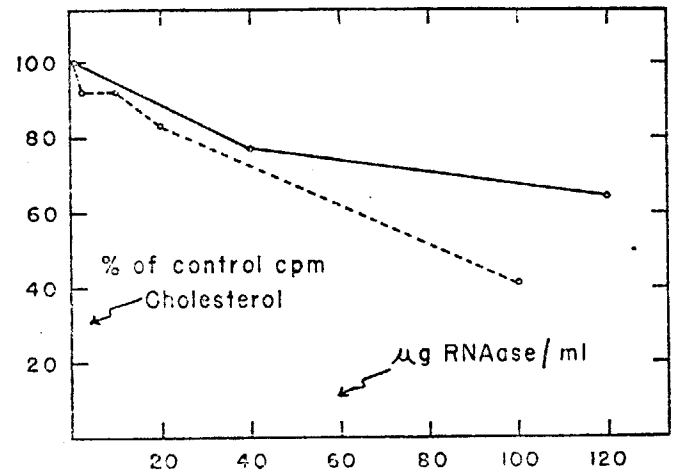

FIG. 2. Effect of ribonuclease. The effect of increasing concentrations of ribonuclease upon the formation of cholesterol *in vitro* is shown in two experiments. Supernatant fluid, obtained by centrifugation of homogenates at 700 × *g*, was incubated under conditions similar to the "Buffer medium" series of Table I, except that ribonuclease was added at the start of incubation in the amounts shown. The results are expressed as per cent of the activity obtained in the controls to which no ribonuclease was added.

among the least active. Further, the addition of ribonuclease to the incubation mixture resulted in negligible inhibition at low concentrations, *i.e.* 10 to 15 γ per ml., as shown in Fig. 2.

Experiments in Vivo: Fractionation Following Injection of Acetate-1-C^{14} into Whole Animal—Experiments carried out in the whole animal provided additional support for the findings *in vitro*. Labeled acetate (125 and 25 μc., respectively) was injected intravenously into two rats, and the livers were removed and fractionated after 3 or after 30 minutes. Since the labeled cholesterol was already formed by the time the livers were excised, a fractionation procedure could be employed which was designed to yield cleaner cell particles than in the studies *in vitro* for which concentrated

homogenates were necessary. Accordingly, the livers were homogenized in 10 volumes of 0.25 M sucrose solution, and the crude homogenate was centrifuged at 500 × *g* to eliminate cell débris and nuclei. Fractionation of the resulting supernatant fluid was carried out as before, except that the crude mitochondrial fraction (sediment from centrifugation at 5000 × *g*) was washed by resuspension in ample volumes of sucrose solution and recentrifugation. The "fluffy layer" (11), which appeared under these conditions as a loosely packed pinkish layer on top of the buff-colored mito-

TABLE III

Cholesterol Formation in Cell Fractions in Vivo

In this experiment and two others in which fractionation was carried out at 3 minutes after the injection of labeled acetate, the total recovery in the fractions was found to be 107, 100, and 102 per cent, respectively, of the counts per minute in the unfractionated starting material. The distribution of the counts among the fractions was similar, in all instances, to that shown above. The percentage recovery in the 30 minute experiment was not determined.

Tissue preparation	Cholesterol					
	Per cent of total c.p.m.		Per cent by weight		C.p.m. per mg.	
	3 min.	30 min.	3 min.	30 min.	3 min.	30 min.
5,000 × *g* sediment (washed)	5	3	2	3	696	636
30,000 × *g* "	52	52	38	49	618	644
60,000 × *g* "	32 } 91	30 } 90	38 } 84	28 } 84	400	656
105,000 × *g* "	7	8	8	7	400	708
105,000 × *g* supernatant	4	7	16	14	128	284

chondrial sediment, was carefully aspirated and added to the supernatant fluid for subsequent refractionation with the microsomal materials (11–13).

In both livers 90 per cent of the total counts per minute in "Cholesterol" was recovered in the three microsomal fractions, chiefly in the large microsomes as before (Table III). It is known that during the first few minutes after the injection of C^{14}-acetate the labeled non-saponifiable lipide which precipitates with digitonin is a mixture of cholesterol and other largely unidentified steroids (14, 15). Since it is highly probable that these radioactive companion substances are cholesterol precursors, in the present short term experiments *in vivo* they are included in the fraction designated "Cholesterol."

The total "cholesterol" content of the fractions again approximately paralleled the radioactivity; over 80 per cent was in the microsomes. Unlike the previous experiment, only a negligible amount was present in the now

less contaminated mitochondria (Table III). The specific activity of the "cholesterol" was significantly lower in the soluble fraction than in the particulates. It was high in both mitochondria and microsomes and similar in both. The results at 3 minutes and at 30 minutes were in reasonably good agreement.

Inhibitors and Requirements—That the mitochondria did not play an essential rôle in cholesterol biosynthesis was further suggested by the observation that potassium cyanide, in concentrations sufficient to inhibit

TABLE IV

Effect of Cyanide and Dinitrophenol upon Cholesterol Formation by Rat Liver Preparations

Conditions similar to the "Buffer medium" series in Table I, except that KCN or sodium 2,4-dinitrophenoxide was added in the concentrations shown. Four separate experiments involving KCN and three involving dinitrophenol are tabulated.

Tissue preparation	Added KCN			KCN:control
	None	0.001 M	0.002 M	
	c.p.m.	*c.p.m.*	*c.p.m.*	*per cent*
700 × *g* supernatant	359	324	265	90, 74
700 × *g* "	532		482	91
9000 × *g* "	195	196		100
9000 × *g* "	263	249	240	95, 91
	Added dinitrophenol			DNP:control
	None	5×10^{-6} M	1×10^{-4} M	
500 × *g* supernatant	562	529	423	94, 75
9000 × *g* "	389	347	334	89, 86
9000 × *g* "	267	230	232	86, 87

cytochrome oxidase (16), and dinitrophenol, in amounts sufficient to uncouple oxidative phosphorylation (17), did not block the reaction *in vitro* (Table IV). Since cytochrome oxidase (18) and the enzymes which mediate oxidative phosphorylation are known to be localized in the mitochondria (18, 19), at least the major mitochondrial function of providing energy through oxidative pathways appeared not to be required.

In the absence of mitochondria, glycolysis might be expected to supply the requisite energy, an idea further supported by the requirement of the system for magnesium ions (2) and by its sensitivity to iodoacetate (Table V). More direct evidence for the dependence of the system upon glycolysis is presented in Table VI. To eliminate endogenous glycogen, saliva was introduced into the mixture at the start of incubation. A striking re-

duction in activity resulted. The effect of the saliva was completely reversed by the addition of hexose diphosphate (HDP). For best results, Versene was included in the mixture; its effectiveness was due presumably to counteraction of impurities in either the saliva or the hexose diphosphate. Since the formation of cholesterol from acetate is primarily a re-

TABLE V

Effect of Potassium Iodoacetate on Cholesterol Formation

Conditions similar to the "Buffer medium" series in Table I, except that potassium iodoacetate was added in the concentrations shown. Two separate experiments are tabulated.

Tissue preparation	Added iodoacetate			
	None	2×10^{-5} M	2×10^{-4} M	2×10^{-3} M
	c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*
9000 × *g* supernatant	376	178	44	2
9000 × *g* "	246	169	51	0

TABLE VI

Relation of Cholesterol Formation to Glycolysis

Conditions similar to the "Buffer medium" series in Table I, except that 0.01 M glutathione was added to the homogenization medium in the second and third of the three experiments tabulated below. Additions to all beakers were 0.016 M hexose diphosphate, 0.001 M disodium Versenate, and 0.3 ml. of saliva, as indicated, in a total volume of 2.6 ml.

Tissue preparation	Additions to incubation mixture		
	None	Saliva + Versene	Saliva + HDP + Versene
	c.p.m.	*c.p.m.*	*c.p.m.*
9000 × *g* supernatant	491	26	458
9000 × *g* "	359*	34	369
9000 × *g* "	660	7	715

* Contained Versene and HDP.

ductive synthesis, the function of glycolysis is probably 2-fold: to provide a continuing supply of reduced diphosphopyridine nucleotide ($DPNH^+$), and to provide energy in the form of adenosine triphosphate (ATP). The requirement of the system for DPN has been shown previously (2). A dependence upon the adenylic acid system was demonstrated by experiments in which the soluble fraction was dialyzed, then recombined with microsomes and incubated under the usual conditions. The activity was negligible unless ATP was added (Table VII).

In a glycolysis-dependent system, in the absence of mitochondria, it seemed possible that the reaction would proceed equally well or better anaerobically, but such was not the case (Table VIII). When incubated under nitrogen, the level of radioactivity found in the cholesterol was only

TABLE VII

Effect of ATP on Cholesterol Formation

Supernatant fluids derived from centrifugation at 9000 × *g* were centrifuged for 30 minutes at 105,000 × *g*. The resulting supernatant fluids were dialyzed at 4° against 25 volumes of buffer medium containing 0.01 M glutathione. The sediments, containing microsomes, were resuspended in the dialyzed supernatant fluids, and the mixture was incubated under the usual conditions (Table I). Two separate experiments are shown.

Tissue preparation	Time of dialysis	Added ATP	
		None	0.0016 M
	hrs.	*c.p.m.*	*c.p.m.*
105,000 × *g* sediment + supernatant	4	1	164
105,000 × *g* " + "	14	1	89

TABLE VIII

Effect of Incubation under Nitrogen on Cholesterol Formation

Conditions similar to the "Buffer medium" series of Table I. For the anaerobic experiments, prior to incubation, Warburg flasks were gassed with nitrogen for 20 minutes with manual shaking in an ice bath, and labeled acetate was then tipped in from a side arm. The nitrogen was washed through Fieser's solution to reduce the O_2 as much as possible. Two separate experiments are shown.

Tissue preparation	Conditions of incubation		
	Aerobic	Anaerobic	Ratio, anaerobic-aerobic
	c.p.m.	*c.p.m.*	*per cent*
9000 × *g* supernatant	308	55	18
9000 × *g* "	283	57	20

about 20 per cent of that obtained under air. The Q_{O_2} values of such preparations were one-eighth to one-tenth those of unfractionated starting material; *i.e.*, supernatant fluid derived from centrifugation at 700 × *g*.

In regard to the stability of the cholesterol-forming enzymes, it is noteworthy that the supernatant fluid derived from centrifugation at 9000 × *g* retained its activity undiminished for at least 17 days when frozen rapidly and stored at −78°.

DISCUSSION

From the above evidence it appears probable that the microsomes are the principal locus of the cholesterol-forming enzymes within the cell: cholesterol synthesis *in vitro* proceeded effectively in the presence of microsomes plus soluble cell constituents, and *in vivo*, following the injection of labeled acetate, nearly all of the C^{13}-cholesterol appeared in the microsomal fraction.

Some incorporation invariably occurred when the mitochondrial fraction was incubated with the soluble fraction; this seems best explained on the basis of contamination of the mitochondria with microsomes, for which there is the following evidence: (1) identification with the phase-contrast microscope; (2) RNA analysis, which showed a level several-fold higher than that found in mitochondria prepared under optimal conditions for purity (20); (3) the finding of much more cholesterol in the unwashed mitochondria obtained from the concentrated homogenates of the experiments *in vitro* than in the relatively clean mitochondria of the studies *in vivo*. In the experiments *in vitro* of Table II, unwashed mitochondria were used preferentially because of the danger of misleading results due to possible elution or destruction of essential factors by washing; in such a case there would be uncertainty as to whether the activity were really negligible in this fraction or had merely been destroyed by our handling of it. The evidence indicates that it is really negligible, since, relative to the amount of protein present, the large microsomes were 4 times more efficient than the crude mitochondrial fraction in effecting conversion of acetate to cholesterol (Fig. 1).

Cholesterol formation from acetate has been reported by other investigators to take place in particle-free preparations of rat liver which contained the soluble components of the homogenate plus an aqueous extract of the mitochondria. The conditions under which the mitochondrial fraction was prepared, however, were such that a major portion of the large microsomes must also have been included; *i.e.*, centrifugation at 33,000 × *g* for 40 minutes (21, 22). It seems likely, therefore, that the activity obtained in this manner should similarly be attributed to the presence of the microsomes.

In the experiments *in vivo*, over 90 per cent of the radioactive cholesterol was recovered in the microsomes at even an early interval after the injection of labeled acetate. This finding strongly favors the microsomes as the site of synthesis, or at least of its final stages. In general, one expects the product of a reaction to exhibit its maximal specific activity at the site where it is synthesized. On this basis the soluble fraction could be ruled out, since the specific activity as well as the total activity of the cholesterol derived from it was considerably lower than that from the other fractions.

The mitochondrial fraction could be ruled out because, although its cholesterol had a specific activity equal to that obtained from the microsomes, its total activity was insignificant. Probably the actual cholesterol content of the mitochondria is negligible, and the small amount found in this fraction, about 5 per cent or less of the total, is principally due to traces of microsomes still remaining; hence, its specific activity would be the same as that of the large microsomes.

The microsomes are not a homogeneous material, either as to composition (13, 23–25) or to morphology. By electron microscopy they seem to be largely composed of fragments of a system of double membranes, or vesicular or tubular structures which have been observed to occur in the cytoplasm (26–28). Closely associated with these membranous structures are dense small granules (29, 30) which consist principally, if not entirely, of ribonucleoprotein (31, 32). Other small cytoplasmic structures may also be included in this fraction (33), but have not been positively identified.

We found the cholesterol concentration to be higher in the larger microsomes, in which cholesterol synthesis was also more active, and the RNA concentration to be higher in the smaller ones. The importance of the ribonucleoprotein particles in the incorporation of amino acids into proteins has been clearly demonstrated (31). This reaction is strongly inhibited by ribonuclease (34, 35); 2 γ per ml. reduced incorporation to 50 per cent of the control level, and 10 γ per ml. to 15 per cent or less.[2] Since ribonuclease at these concentrations had a negligible effect upon cholesterol synthesis (Fig. 2), and since the RNA-rich small microsomes exhibited a low capacity to form cholesterol, the RNA particles appear not to be involved with cholesterol synthesis. Rather, the evidence suggests that the particulates concerned are the larger membranous or vesicular structures or other elements yet unidentified.

At least two of the contributions of the soluble fraction of the cell to the cholesterol-forming system can be specified. The enzymes which mediate glycolysis, which we have demonstrated to be required by the *in vitro* system, are reported to occur in the soluble portion of liver homogenates (36). Secondly, the activation of acetate, presumed to be the initial step in the reaction sequence, appears to take place entirely in the soluble fraction; in preliminary studies acetate activation in this fraction was equal to that occurring in the unfractionated material, whereas intact particles contained negligible activity.[3] Where the soluble enzymes really occur within the living cell is uncertain; some may be actually in solution in the cytoplasmic

[2] Unpublished data kindly supplied by Dr. Elizabeth B. Keller of the Collis P. Huntington Memorial Laboratories.

[3] For these data we are much indebted to Dr. Mary Ellen Jones of the Biochemical Research Laboratories of the Massachusetts General Hospital.

matrix; others may be localized within the particulates and released during homogenization.

Recent studies by Tchen and Bloch have shown that the cyclization of squalene to form lanosterol and the conversion of lanosterol to cholesterol take place *in vitro* in the presence of microsomes and soluble cell components (37).

When Strittmatter and Ball demonstrated cytochrome *m* in the microsomes, they suggested that these particles would be the logical site for a reductive synthesis such as that of cholesterol because of the particular properties of this cytochrome (38, 39). The present evidence indicates that the microsomal enzymes do play an important rôle, although its exact nature remains to be further elucidated.

SUMMARY

For the biosynthesis of cholesterol from acetate to occur *in vitro*, both microsomes and soluble cell constituents are required. Other cellular structures are not necessary.

In vivo, over 90 per cent of newly formed cholesterol obtained at short time intervals after the injection of labeled acetate is in the microsomal fraction.

The microsomes contain over 80 per cent of the total cytoplasmic cholesterol.

The *in vitro* synthesis is dependent upon a glycolytic substrate, ATP, and TPN. In addition, there is a requirement for oxygen, although cyanide and dinitrophenol fail to block the reaction. The glycolytic enzymes, the coenzyme-activating enzyme, and probably some others contributing to the conversion of acetate to cholesterol are in the soluble fraction.

The microsomal fraction contains the particles principally concerned with cholesterol synthesis. Within the microsomes, the larger lipide-rich membranous or vesicular structures, or other elements still unidentified, rather than the smallest granules or ribonucleoprotein particles, appear to be the most probable locus of the cholesterol-forming enzymes.

We are grateful to Dr. Joseph C. Aub for encouragement, to Dr. Fritz Lipmann for helpful suggestions regarding the demonstration of glycolysis, to Dr. Roger Jeanloz for aid in purifying the hexose diphosphate, and to Dr. Elizabeth B. Keller for valuable criticism and much good advice.

Addendum. When microsomes plus soluble cell components are incubated anaerobically with acetate-$1,2-C^{14}$, isolated squalene accumulates. In a typical experiment, 3600 c.p.m. were obtained in squalene and only 7 c.p.m. in cholesterol. A similar preparation incubated aerobically yielded 48 c.p.m. in squalene and 1455 c.p.m. in cholesterol. Thus oxygen was not required for the conversion of acetate to squalene, but was essential for the conversion of squalene to cholesterol. Neither of these two steps occurred in the absence of a pyridine nucleotide and a glycolytic substrate.

BIBLIOGRAPHY

1. Bucher, N. L. R., *J. Am. Chem. Soc.*, **75**, 498 (1953).
2. Frantz, I. D., Jr., and Bucher, N. L. R., *J. Biol. Chem.*, **206**, 471 (1954).
3. Sable, H., *Biochem. Preparations*, **2**, 53 (1952).
4. Nieft, M. L., and Deuel, H. J., Jr., *J. Biol. Chem.*, **177**, 143 (1949).
5. Sperry, W. M., and Webb, M., *J. Biol. Chem.*, **187**, 97 (1950).
6. Cornall, A. G., Bardawill, C. J., and David, M. M., *J. Biol. Chem.*, **177**, 751 (1949).
7. Scott, J. F., Fraccastoro, A. P., and Taft, E. B., *J. Histochem. and Cytochem.*, **4**, 1 (1956).
8. Schneider, W. C., and Hogeboom, G. H., *Cancer Res.*, **11**, 1 (1951).
9. Huseby, R. A., and Barnum, C. P., *Arch. Biochem.*, **26**, 187 (1950).
10. Kennedy, E. P., and Lehninger, A. L., *J. Biol. Chem.*, **179**, 957 (1949).
11. Schneider, W. C., *J. Histochem. and Cytochem.*, **1**, 212 (1953).
12. Muntwyler, E., Seifter, S., and Harkness, D. M., *J. Biol. Chem.*, **184**, 181 (1950).
13. Novikoff, A. B., Podber, E., Ryan, J., and Noe, E., *J. Histochem. and Cytochem.*, **1**, 27 (1953).
14. Schwenk, E., and Werthessen, N. T., *Arch. Biochem. and Biophys.*, **40**, 334 (1952).
15. Dayton, S., Mosbach, E. H., and Kendall, F. E., *Proc. Soc. Exp. Biol. and Med.*, **84**, 608 (1953).
16. Green, D. E., Mechanisms of biological oxidations, Cambridge (1941).
17. Loomis, W. F., and Lipmann, F., *J. Biol. Chem.*, **173**, 807 (1948).
18. Hogeboom, G. H., Schneider, W. C., and Striebich, M. J., *Cancer Res.*, **13**, 617 (1953).
19. Kidley, W. W., and Kidley, R. K., *J. Biol. Chem.*, **191**, 485 (1951).
20. Kuff, E. L., and Schneider, W. C., *J. Biol. Chem.*, **206**, 677 (1954).
21. Rabinowitz, J. L., and Gurin, S., *Biochim. et biophys. acta*, **10**, 345 (1953).
22. Rabinowitz, J. L., and Gurin, S., *J. Biol. Chem.*, **206**, 307 (1954).
23. Chantrenne, H., *Biochim. et biophys. acta*, **1**, 437 (1947).
24. Barnum, C. P., and Huseby, R. A., *Arch. Biochem.*, **19**, 17 (1948).
25. Peterman, M. L., and Hamilton, M. G., *Cancer Res.*, **12**, 373 (1952).
26. Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, **81**, 233 (1945).
27. Slautterback, D. B., *Exp. Cell Res.*, **5**, 174 (1953).
28. Palade, G. E., and Siekevitz, P., *Federation Proc.*, **14**, 262 (1955).
29. Palade, G. E., *J. Biophys. and Biochem. Cytol.*, **1**, 59 (1955).
30. Sjöstrand, F. S., and Hanzon, V., *Exp. Cell Res.*, **7**, 393 (1954).
31. Littlefield, J. W., Keller, E. B., Gross, J., and Zamecnik, P. C., *J. Biol. Chem.*, **217**, 111 (1955).
32. Peterman, M. L., Mizen, N. A., and Hamilton, M. G., *Cancer Res.*, **13**, 372 (1953).
33. Hogeboom, G. H., and Kuff, E. L., *Federation Proc.*, **14**, 633 (1955).
34. Zamecnik, P. C., and Keller, E. B., *J. Biol. Chem.*, **209**, 337 (1954).
35. Allfrey, V., Daly, M. M., and Mirsky, A. E., *J. Gen. Physiol.*, **37**, 157 (1953).
36. LePage, G. A., and Schneider, W. C., *J. Biol. Chem.*, **176**, 1021 (1948).
37. Tchen, T. T., and Bloch, K., *J. Am. Chem. Soc.*, **77**, 6085 (1955).
38. Strittmatter, C. F., and Ball, E. G., *Proc. Nat. Acad. Sc.*, **38**, 19 (1952).
39. Strittmatter, C. F., and Ball, E. G., *J. Cell. and Comp. Physiol.*, **43**, 57 (1954).

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OBSTETRICS

Drug preservatives and uterine contractility

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IN VITRO methodology for the study of human uterine contractile patterns has been used since the beginning of this century.[1] It has proved to be a simple and useful method for observation of the direct, local response of uterine smooth muscle, pregnant and nonpregnant, to applied stimuli. In many instances in vitro evaluation has been used to compliment in vivo investigation, and some attempts have been made to extrapolate the results obtained with isolated tissues to the living subject. With most stimuli such an extrapolation is probably valid. However, the majority of commercially available pharmaceutical medicaments are not dispensed as single entities, but contain additional chemicals to enhance solubility, minimize decomposition, ensure sterility, or stabilize hydrogen ion concentration. Inclusion of these substances in the pharmaceutical preparations under study raises the question as to their effect on the spontaneous contractile patterns of isolated human uterine tissues. The present study was undertaken to evaluate the effect of five chemicals frequently added, as preservatives, to pharmaceutical medicaments. Chlorobutanol, alcohol, phenol, sodium acetate, and propylene glycol were selected for study.

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Methods

Gravid and nongravid uterine muscle strips were excised at the time of cesarean section or following hysterectomy for benign con-

ditions in premenopausal women. Each strip was uniformly suspended in a chamber containing oxygenated Locke's solution (modified by addition of 0.84 millimole of magnesium chloride per liter of solution) and so arranged as to actuate a light, standardized, ink-writing lever held by gravity against a continuously recording kymograph. Aeration of the strip and mixing of the solution were achieved by bubbling oxygen gas through the modified Locke's solution. Temperature of the system was maintained at 37° C.

Each strip was medicated with but one of the study agents to negate synergistic or antagonistic responses incident to retention of previously studied chemicals despite repetitive washings. In view of the marked variability in spontaneous contractile patterns displayed by isotonically contracting smooth muscle samplings, each strip was employed as its own control. Thus, only after recording a control pattern was the tissue exposed to the test agent. Three different parameters were employed for comparison: magnitude of contraction, or the average height in millimeters of the contraction waves as they appeared on the kymograph; frequency of contraction; and "tonus," defined as the average height of the recording stylus from the base line on the kymograph at the end of a contraction-relaxation complex.

Results

In Figs. 1 through 5, representative tracings of the myometrial responses to increasing concentrations of the five agents selected for study are presented. The arrows indicate the moment of addition of the test substance to the tissue chamber and the numbers depict the resultant concentration of the agent in each milliliter of bath solution.

On increasing the concentration of chlorobutanol within the tissue bath a decrease in frequency of contraction, a diminution in height of contraction, and a reduction in tone were observed with nongravid myometrial strips. With pregnant samplings the contractile pattern was similarly disturbed. At a concentration of 20 μg per milliliter of bath fluid spontaneous activity could be completely inhibited. The response patterns of isolated human myometrial samplings to medication with chlorobutanol, a chloroform derivative commonly added to oxytocic, estrogenic, progestational, and androgenic commercial preparations, is typical of a depressant response.[2]

On addition of sodium acetate to the tissue bath, nonpregnant and pregnant human uterine strips displayed a decrease in the frequency of contraction. Sodium acetate is added to different commercial preparations (vitamins, oxytocics) because of its buffering action.

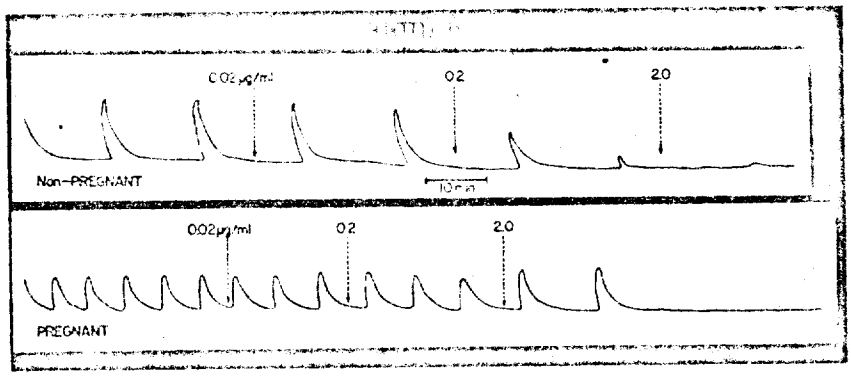

Fig. 1. Effect of chlorobutanol on spontaneous contractile patterns of isolated human myometrial tissues. The arrows indicate the moment of addition of the chemical to the tissue chamber and the numbers refer to the resultant concentration of the drug in micrograms per milliliter of bath solution.

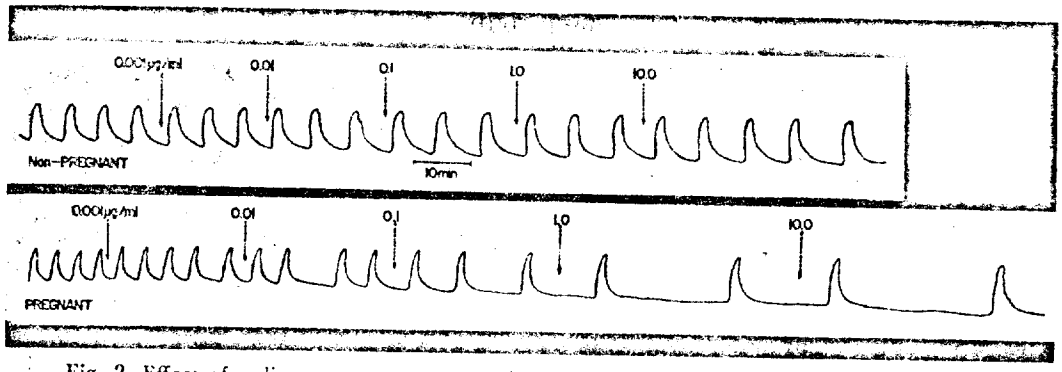

Fig. 2. Effect of sodium acetate on contractile patterns of nonpregnant and pregnant human myometrial smooth muscle in vitro. The arrows indicate the moment of addition of the chemical to the tissue chamber and the numbers refer to the resultant concentration of the agent in micrograms per milliliter of bath solution.

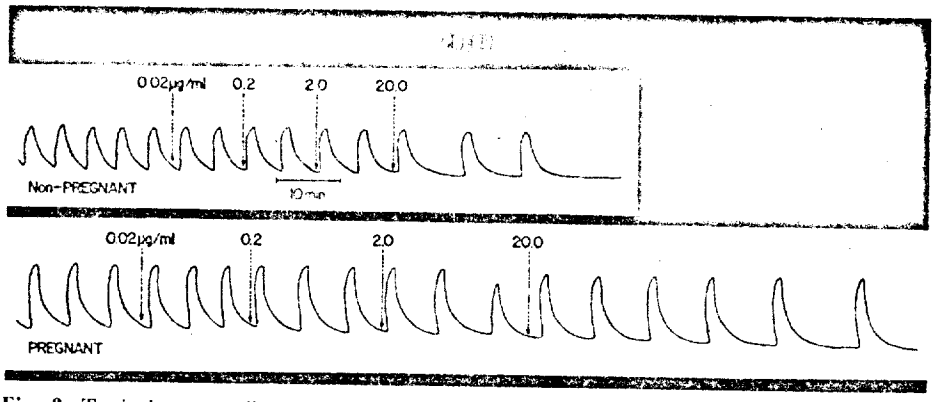

Fig. 3. Typical contractile response patterns of isolated human myometrial samplings to increasing concentrations of phenol within the tissue chamber. Moment of addition of the agent to the modified Locke's solution and the resultant tissue bath concentration in micrograms per milliliter are depicted.

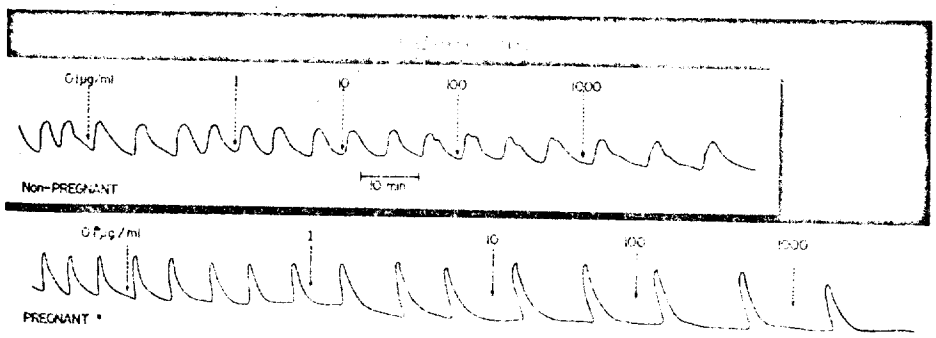

Fig. 4. Response patterns of nonpregnant and pregnant myometrial strips contracting isotonically to medication with propylene glycol. Moment of addition of the agent to the modified Locke's solution and the resultant tissue bath concentration in micrograms per milliliter are depicted.

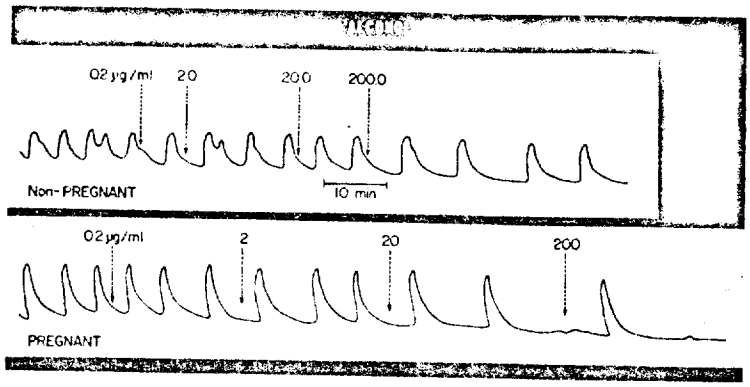

Fig. 5. Response patterns of nonpregnant and pregnant human myometrial strips contracting isotonically to medication with alcohol. Moment of addition of the agent to the modified Locke's solution and the resultant tissue bath concentration in micrograms per milliliter are depicted.

Phenol is the oldest of the antiseptic agents being introduced for this purpose by Lister, in 1867. It is found in a variety of commercial preparations including vitamins, vaccines, and opium derivatives. On addition of this agent to the muscle tissue bath decreases in frequency and tone were observed.

Propylene glycol, commonly employed as a preservative in commercial preparations (tranquilizers, antihypertensive agents), produced a definite slowing of the rate of contraction and a slight decrease in tone of the contractile pattern of both pregnant and nonpregnant human myometrial tissues.

On addition of alcohol to isolated human uterine samplings decreases in frequency and tone were observed. Alcohol is frequently added as a vehicle in a variety of clinically useful drugs such as cardiotonics, analgesics, oxytocics, and vitamins.

Comment

The contractile patterns of isolated human myometrial tissues were altered by medication with each of the five agents included in this report.

Disturbance in pattern incident to the addition of phenol to the tissue bath is not surprising since this agent is a well-known protoplasmic poison. Phenol is capable of altering the physical and chemical properties of protein at very dilute concentrations apparently because the protein-phenol complex formed during the denaturation process is relatively unstable. Following alteration of peptide linkages in the attached protein, the toxic agent escapes from the denatured tissue and is capable of attacking other protein molecules with repetition of the process.[3]

In the present study a decrease in the spontaneous contractile activity of uterine myometrial samplings was observed on addition of alcohol to the tissue chamber. A disturbance in the contractile properties of muscle cells incident to medication with ethanol is not a unique response of uterine smooth muscle but has also been observed with cardiac and skeletal muscles in vitro. With these tissues a shortening of the action potential[4] and a depolarization of the membrane[5] have been recorded suggesting that the mode of action of alcohol is due, at least in part, to a disturbance in membrane dynamics.

An inhibitory effect of propylene glycol on the contractility of the isolated ileum of the guinea pig and the duodenum of the rat has been described by Laroche.[6] The present observations indicate that propylene glycol is also capable of altering the contractile patterns of human uterine samplings in vitro.

In a brief communication in 1928, Terasaka[7] published his observations on the inhibitory effect of chlorobutanol on the spontaneous activity of excised strips of rabbit

uterine muscle. In 1947, Riley[8] confirmed the in vitro studies of Terasaka and noting that the agent was capable of depressing the rabbit uterus in vivo suggested that the reported inhibitory effect of some commercial preparations on myometrial activity might be due to inclusion of this germicidal agent. In 1950, Krantz,[9] studying the effect of corpus luteum aqueous solutions, reported that while chlorobutanol was depressant to isolated segments of guinea pig uteri, it was without effect on the intact uterus at a dosage level of five milligrams per kilogram of body weight. The present studies indicate that chlorobutanol is depressant to human myometrial tissues in vitro.

From the data presented, it is apparent that inclusion of antiseptic, germicidal, and preservative agents in commercial preparations complicate in vitro appraisal of pharmaceutical medicaments. The data suggest that the myogenic action of each substance included in impure preparations must be equated in order to assess the direct, local response of isolated human uterine tissues to the particular ingredient in the preparation under study. In addition, the biological half-life and myogenic effect of the degradation products of each substance included in the test medicament would need to be equated for accurate extrapolation of in vitro results to the intact organism.

At the present state of our knowledge we believe that in vitro methodology offers an excellent study technique for evaluation of muscle mechanics incident to drug action, but its limitations must be considered in attempts to extrapolate in vitro observations to intact preparations. We suggest that in vitro methodology should be employed as an adjunctive to, rather than a substitute for, in vivo appraisal of the contractile response of myometrial tissue to pharmaceutical medicaments.

It is unfortunate that alcohol and propylene glycol disturb the spontaneous contractile patterns of isolated human uterine smooth muscle samplings since many drugs are not readily soluble in the various salt solutions employed in in vitro investigation. Use of such vehicles to enhance solubility of the test agent in the artificial medium serves to complicate evaluation but should not negate the validity of controlled experimentation.

Conclusion

Chlorobutanol, alcohol, phenol, sodium acetate, and propylene glycol are depressant to isotonically contracting human uterine smooth muscle in vitro.

REFERENCES

1. Kehrer, E.: Arch. Gynäk. 81: 160, 1907.
2. Johnson, W. L., McGaughey, H. S., Jr., Scoggin, W. A., Wilson, L. A., Jr., and Thornton, W. N., Jr.: Am. J. Obst. & Gynec. 86: 296, 1963.
3. Goodman, L. S., and Gilman, A.: The pharmacological basis of therapeutics, ed. 2, New York, 1960, The Macmillan Company.
4. Gimeno, A. L., Gimeno, M. F., and Webb, J. L.: Am. J. Physiol. 203: 194, 1962.
5. Knutson, E.: Acta physiol. scandinav. 52: 242, 1961.
6. Laroche, M. J., Maziere, M., and Cottart, A.: Therapie (Paris) 18: 503, 1963.
7. Terasaka, M.: J. Chosen M. A. 85: 168, 1928.
8. Riley, G. M.: Am. J. Obst. & Gynec. 54: 40, 1947.
9. Krantz, J. C., Jr., Briant, H. H., and Carr, C. J.: Surg. Gynec. & Obst. 90: 372, 1950.

Cytochemical Hematological Studies on Subjects Exposed to Occupational Hazards of Intoxication by Acetone and Acetic Acid

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Hematological cytochemical tests have been carried out on 2 groups of subjects employed as skilled workers in a large industrial complex of Northern Italy manufacturing, among other products, cellulose acetate filaments.

Workers in the first group were exposed to acetone hazards, while those in the second group were exposed to acetic acid intoxication; some of these workers had already suffered, a long time ago, from some episode of subacute intoxication, which had been well overcome and after which they resumed their previous jobs.

In the ambient air of the various work sections, for example in the solutions section of the first group and in the acetylation section of the second group, the concentrations of toxic products were as follows: about 0.20-0.36 mg/l acetone, and about 0.06-0.66 mg/l acetic acid.

The object of the present study is to describe hematological cytochemical tests carried out on peripheral blood smears, in order to determine eventual alterations caused by exposure to the occupational hazards mentioned above.

Methods and Results

Smears of peripheral blood were taken from fasting subjects. Prior to adequate fixation, the following staining procedures were carried out:

1) Panoptic staining with May-Grunwald and Giemsa reagents.
2) Lipid staining with Sudan Black B, according to Sheehan and Storey.
3) Staining of polysaccharides with periodic acid and Schiff's reagent, according to Hotchkiss.
4) Staining of ribonucleic acid by the Unna-Pappenheim method.
5) Staining of desoxyribonucleic acid by the Feulgen method.
6) Staining of peroxidase diastases by the Sato method.
7) Staining of peroxidase diastases by the Undritz method.
8) Staining of oxidase diastases by the Winckler and Schultze technique.

The leucolipid coefficient of blood (CLS) was determined; the method used and complete technical details are described in a previous study by Granata.

Similarly, two other coefficients were introduced: the leucoxidase (CLO) and the leucoperoxidase (CLP) coeffients of peripheral blood. To calculate such coefficients, as well as CLS, a proportionality factor of 3 was assigned to cellular elements exhibiting a maximum reactive intensity, (reactivity) a factor of 2 to elements of medium reactivity, and a factor of 1 to elements of minimum reactivity. The number of cellular elements is multiplied by the corresponding factor of proportionality, the 3 products obtained are added, and the sum is divided by 100. Thus, the formulas are as follows:

$$\text{Leucoxidase coefficient (CLO)} = \frac{3(Mx) + 2(Md) + 1(mn)}{100}$$

$$\text{Leucoperoxidase coefficient (CLP)} = \frac{3(Mx) + 2(Md) + 1(Mn)}{100}$$

For example, if, in the examination of a blood smear, 38 out of 100 elements are found with a maximum oxidase reaction, 44 with a medium oxidase reaction, and 18 with a minimum oxidase reaction, then we get:

$$CLO = \frac{3(38) + 2(44) + 1(18)}{100} = \frac{114 + 88 + 18}{100} = 2.20$$

Theoretically, the above coefficients can vary from a minimum of 1 (when all elements have a minimum reaction intensity) to a maximum of 3 (when all elements examined have a maximum intensity).

The introduction of these coefficients is useful in making an approximate quantitative estimate, when it is impossible to perform a histospectrophotometric examination.

In the subjects examined, the duration of exposure to the occupational hazard varied from a minimum of 2 to a maximum of 25 years for acetic acid.

The experimental results obtained are given below:

First Group - Subjects Exposed to Acetone Hazards

1) Panoptic staining according to May-Grunwald-Giemsa. As can be seen from Table 1, increases are noted in the lympho-monocytic percentage values. In 7 out of 9 cases, the majority of monocytes exhibited sparse granulations on the nucleus, of dark blue, almost black color (Fig. 1). All cases of relative monocytosis included such a finding.

Abnormal forms have not been noted, either of the red or white series. Arneth's formula was found to be normal in all cases.

Table 1. Subjects exposed to acetone hazards. Peripheral blood findings. May-Grunwald-Giemsa panoptic staining.

Cases	1°	2°	3°	4°	5°	6°	7°	8°	9°
nome (1)	A B	M A	M F	C A	F P	D F	S A	R G	P G
età (2)	55	47	52	57	35	60	36	47	34
anni di rischio (3)	14	13	25	18	11	14	12	12	2
Formula leucocitaria (4) neutrofili (5)	54	44	59	68	60	55	58	56	55
eosinofili (6)	2	2	—	—	2	2	—	—	2
basofili (7)	4	—	—	—	—	—	2	—	4
linfociti (8)	30	50	35	26	34	31	24	28	33
monociti (9)	10	4	6	6	4	12	16	16	6
granulazioni monocitarie (10)	Pres. (11)	ass. (12)	ass.	pres.	pres.	pres.	pres.	pres.	pres.

1 - Name
2 - Age
3 - Years of exposure
4 - Leucocytic formula
5 - Neutrophils
6 - Eosinophils
7 - Basophils
8 - Lymphocytes
9 - Monocytes
10 - Monocytic granulations
11 - Present
12 - Absent

2) Sudan Black B staining of lipids, according to Sheehan and Storey.

The behavior of Sudanophil leucocytic granules can be seen from Table 2. It should be recalled that the normal value of the blood Leucolipid coefficient (CLS) varies only from 2.53 to 2.81; thus, a decrease of the granulocytic lipid stock is evident in 7 of the 9 cases examined.

Table 2. Acetone hazards; percentage values of granulocytic lipids and of the leucolipid coefficient.

Cases	1°	2°	3°	4°	5°	6°	7°	8°	9°
Granulociti a reazione (1) massima (2)	12	72	20	67	51	30	12	37	13
media (3)	59	20	61	21	38	56	53	45	60
minima (4)	29	8	18	12	11	14	20	18	67
Coeff. leuc. San. (CLS) (5)	1,83	2,61	2,90	2,55	2,40	2,16	1,82	2,19	1,86

1 - Granulocytes reaction
2 - Maximum
3 - Medium
4 - Minimum
5 - Leucolipid coefficient of blood (CLS)

3) Staining of polysaccharides with periodic acid and Schiff's reagent according to Hotchkiss .

Normal results were obtained in all cases. The cytoplasm of the granulocytes assumed a red-violet color, the intensity of which corresponded to the coloring of the same elements in a normal blood smear, taken as a "test" of the reaction.

4) Staining of ribonucleic acid according to Unna and Pappenbeim.

Ribonucleic acid was identified in the nucleus and cytoplasm of elements of the white series. The intensity of the reaction gave the following results: normal for cases 1, 2, 3 and 6; mediocre for case 8; weak for cases 4 and 9; very weak for cases 5 and 7

5) Staining of desoxyribonucleic acid by the Feulgen method.

In all cases, the nuclei of leucocytic elements exhibited a chromatic (color) intensity which was lower than in the normal test.

6) Staining of peroxidase diastases by the Sato and Sekjia method.

From Table 3 we can see that the behavior of peroxidase diastases is almost normal, and this opinion is confirmed by the values of the leucoperoxidase coefficient (CLP), which varies from 2 to 2.50 in normal subjects.

Table 3. Acetone hazards; percentage values of peroxidase diastases and leucoperoxidase coefficient. Staining according to Sato.

Casi / Cases		1°	2°	3°	4°	5°	6°	7°	8°	9°
Granulociti a reazione	massima	35	35	40	49	36	63	52	37	28
	media	42	56	43	47	46	27	40	52	52
	minima	23	9	17	4	18	10	8	11	20
Coef. Leuco-Perisid. (CLP)		2,12	2,20	2,23	2,45	2,18	2,03	2,44	2,20	2,08

1 - Granulocytes reaction
2 - Maximum
3 - Medium
4 - Minimum
5 - Leucoperoxidase coefficient (CLP)

7) Staining of peroxidase diastases by the Undritz method.

The presence of granulations with an intense dark brown to blackish color, particularly abundant in some neutrophils and absent in eosinophiles, against the golden background of the leucocytic cytoplasm, deserves special attention. Monocytic are always observed with such large granulations, without any background recorded in the typical Undritz reaction. A typical example of a neutrophil granulocyte with blackish granulations is shown in Fig. 2.

In general, the coloring revealed in this test indicates a reduction of the peroxidase diastase stock, demonstrated in the cytoplasm of leuocytes. The apparent contrast with the results obtained by the Sato method shows once again the greater finesse (accuracy) and specificity of the Graham-Knoll-Undritz reaction.

In summary, a normal peroxidase content with the absence of blackish granulations was found in cases 8 and 9; a discreet slight) peroxidase content, with blackish granulations present, was found in cases 1, 2, 4, 5 and 6; an extremely weak intensity, with very numerous abnormal granulations, sometimes covering 2/3 of the protoplasm, was found in case 7.

8) Staining of oxidase diastases by the Winckler-Schultze technique.

An exaimination of Table 4 shows a reduction of leucocytic oxidases in 6 out of 9 cases. This can be easily deduced from the leucoxidase coefficient (CLO), which in normal cases should range from 2.25 to 2.75.

Table 4. Acetone Hazards. Percentage values of oxidase diastases and leucoxidase coefficient. Staining according to Winckler and Schultze.

Casi	1°	2°	3°	4°	5°	6°	7°	8°	9°
Granulociti a reazione: massima	34	16	31	41	23	68	35	30	54
media	41	56	45	46	50	24	40	46	37
minima	12	28	24	13	27	8	25	24	9
Coef. Leuco-Ossid. (CLO)	2,20	1,88	2,07	2,28	1,96	2,00	2,10	2,06	2,45

1 - Granulocytes reaction
2 - Maximum
3 - Medium
4 - Minimum
5 - Leucoxidase coefficient (CLO)

Second Group - Subjects Exposed to Acetic Acid Intoxication Hazards

1) Panoptic staining according to May-Grunwald-Giemsa.

A relative lymphocytosis was observed in 5 out of 9 cases, and a monocytosis was also noted in 4 cases. Granulations with an intense blackish color, similar to those observed in acetone intoxication, were noted in 5 cases. Basophil granulocytes were found rather frequently, as can be seen in Table 5, which also shows the age and the number of years of occupational exposure to acetic acid of the subjects examined.

Table 5. Acid acetic intoxication hazards. Findings on peripheral blood smears. Staining according to May-Grunwald-Giemsa.

Casi	1°	2°	3°	4°	5°	6°	7°	8°	9°
nome	R C	P Q	P A	A E	M O	D G	P P	B G	B L
anni	39	34	47	47	38	37	46	55	45
anni di rischio	7	10	24	9	9	9	9	25	10
formula leucocitaria: neutrofili	68	61	52	58	58	55	67	68	53
eosinofili	2	2	—	2	2	3	2	2	2
basofili	1	2	—	2	—	1	1	—	2
linfociti	22	31	38	30	24	31	26	24	33
monociti	7	4	10	8	16	10	8	6	10
granul. monocitarie	ass.	pres.	pres.	pres.	pres.	ass.	pres.	ass.	ass.
formul. di Arneth	N	N	N	N	N	→	→	N	→

1 - Name
2 - Age
3 - Years of exposure hazard
4 - Neutrophils
5 - Eosinophils
6 - Basophils
7 - Lymphocytes
8 - Monocytes
9 - Leucocytic formula
10 - Monocytic granulations
11 - Arneth formula

2) Sudan Black B staining of lipids according to Sheehan and Storey.

A pattern similar to the one observed with acetone hazards can be seen from Table 6. A reduction of the sudanophil stock of granulocytes in 7 out of 9 cases can be deduced from the values of the blood leucolipid coefficient (CLS).

In no case, however, do the CLS values reach the minimum values (1.82 - 1.83) noted in the case of acetone hazards.

Table 6. Acetic acid intoxication hazards. Percentage values of granulocytic lipids and of the blood leucolipid coefficient.

Casi Cases		1°	2°	3°	4°	5°	6°	7°	8°	9°
Granulociti a reazione (1)	massima (2)	78	12	47	15	62	46	14	55	30
	media (3)	16	78	40	67	36	48	71	35	54
	minima (4)	6	10	13	18	2	6	15	10	16
CLS		2,72	2,02	2,34	1,97	2,60	2,40	1,99	2,45	2,14

1 - Granulocytes reaction
2 - Maximum
3 - Medium
4 - Minimum

3) Staining of polysaccharides with periodic acid and Schiff's reagent, according to Hotchkiss.

In the cases examined, this test gave an intensity of reaction corresponding to the one observed in the normal test.

4) Staining of ribonucleic acid by the Unna-Pappenhein method.

Although the findings were positive both for the nucleus and the protoplasm of leucocytic cells, the intensity of the nuclear staining was identified to a much lesser extent. This constitutes a normal record,

which was not observed in cases of acetone hazards. The degree of staining can be summarized as follows: normal in cases 2, 4 and 7; weak in cases 1, 3 and 6; very weak in cases 5, 8 and 9.

5) Staining of desoxyribonucleic acid by the Feulgen method.

As in the case of acetone hazards, here also a slight reduction of the specific nuclear staining was observed in all cases.

6) Staining of peroxidase diastases by the Sato-Seikjia method.

Table 7 shows the behavior of peroxidase diastases in the 9 cases examined. Here also, as in the case of acetone hazards, the leucoperoxidase coefficient was within normal limits.

Table 7. Acetic acid intoxication hazards. Percentage values of peroxidase diastases and of the leucoperoxidase coefficient. Staining according to Sato.

Cases	1°	2°	3°	4°	5°	6°	7°	8°	9°
Granuloriti a reazione massima	39	60	30	60	10	54	31	29	27
media	49	29	56	24	48	35	52	58	61
minima	12	11	14	16	12	11	17	13	12
CLP	2,27	2,49	2,16	2,44	2,28	2,43	2,14	2,16	2,15

(1) to (4) - same as in Table 6.

7) Staining of peroxidase diastases by the Undritz method.

Blackish granulations are also present here, as in the case of acetone hazards. The peroxidase content is in general low, and can be summarized as follows: normal in cases 2, 3 and 6; low in cases 1. 4, 5, 7, 8 and 9.

In some cases (5 and 9), the monocytes exhibited large blackish granulations and the absence of a cytoplasmic background.

8) Staining of oxidase diastases by the Winkler-Schultze technique.

In 5 out of 9 cases, the values of leucocytic oxidases were reduced, and in one case were low enough to reduce the leucoxidase coefficient to 1.72 (minumum of acetone hazard 1.88). For more detailed figures, see Table 8.

Table 8. Acetic acid intoxication hazards. Values of oxidase diastases and leucoxidase coefficient.

	Casi	1°	2°	3°	4°	5°	6°	7°	8°	9°
Granulociti a reazione	massima (2)	28	60	58	35	50	26	42	44	13
	media (3)	54	36	34	43	34	20	40	39	57
	minima (4)	18	4	8	22	16	54	18	17	30
CLO		2,10	2,56	2,50	2,13	2,34	1,72	2,24	2,27	1,83

(1) to (4) - same as in Table 6, 7.

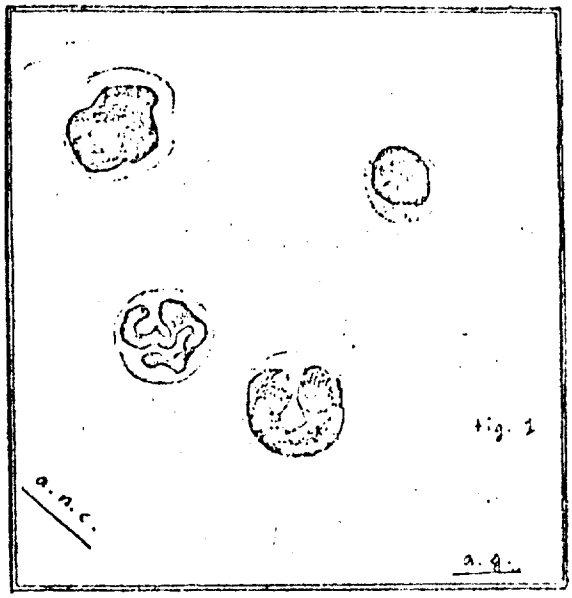

Fig. 1. Acetone hazards. Pappenheim panoptic staining. At right, a monocyte with intensely basophil, abnormal nuclear granulations.

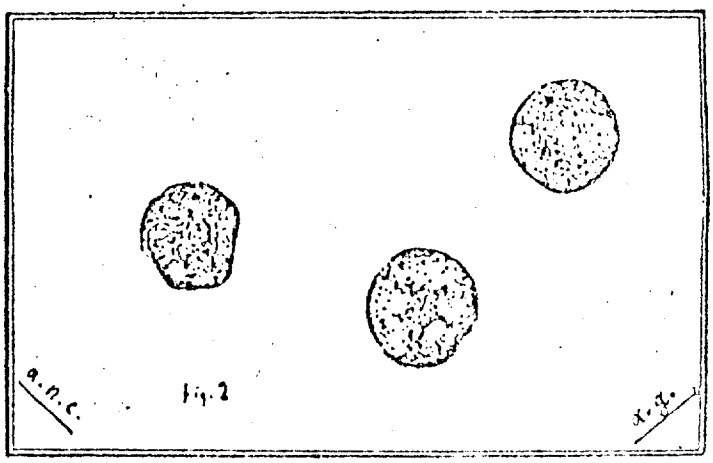

Fig. 2. Acetone hazards. Graham-Knoll-Undritz staining. Neutrophil granulocytes with a low peroxidase content. The blackish granulations described in the text are visible in two elements.

Considerations and Conclusions

a). In the group of workers exposed to acetone hazards, the following deviations from the norm (alterations) have been found: frequent monocytoses and a relative lymphocytosis, a frequent reduction of sudanophilia with a relative lowering (drop) of the blood leucolipid coefficient, and an almost general weakening (decrease) of leucocytic ribo- and desoxyribonucleins (Unna-Pappenheim and Feulgen reactions). On the other hand, finding were normal in regard to polysaccharides and peroxidases identified by Sato's method; in a considerable number of cases, a more or less evident decrease was noted in the content of oxidases and peroxidases, the latter being identified by the Undritz method.

b). In the group of workers exposed to occupational intoxication by acetic acid, alterations have been found which, in most cases, are similar to those observed in the preceding group; however, one has the impression that such alterations are of minor intensity and occur after a longer time of exposure to acetic acid hazards. In this group, we can also find in most cases monocytoses and a relative lymphocytosis, a reduction of sudanophilia, a lowering of the fibo- and desoxyribonucleic acid stocks, and a decrease of oxyperoxidases. On the other hand, polysaccharides and peroxidases detected by Sato's method were normal.

c). In both groups, the presence of highly colored and basophil granulations on the nucleus of some monocytes was noted. Such granulations were identified only by the panoptic staining method, and remained absolutely colorless with the other staining methods used.

d). The reaction of Undritz, in addition to demonstrating a reduction of the cytoplasmic peroxidase content in granulocytes, has indicated the frequent presence of dark-blue granulations, dispersed within the limits of the protoplasm and sometimes covering a large portion of the latter. Such abnormal findings have been detected in both groups of workers examined.

Summary

Hematological cytochemical studies were carried out on two groups of subjects, one group being exposed to the occupational hazards of acetone intoxication, and the other to acetic acid intoxication.

In addition to monocytoses and a relative lymphocytosis, identified by panoptic staining, alterations in the available stock of lipids, nucleic acids and oxyperoxidases (oxydiastases) could be detected by modern hemocytochemical methods.

Bibliography

1. Granata A. Studies on granulocyte lipids in silicotuberculosis, Folia Medica 38: 26-36, 1955 (Italian)

2. Nunziante Cesaro A., Granata A. Hematological cytochemical studies on some spreading occupational diseases, Folia Medica 36: 861-80, 1953 (Italian)

3. Undritz E. A modification of the Knoll peroxidase reaction for the determination of monocytes, Verhandl. Schw. Naturforsch. Ges. 121: 20, 1941 (German)

ISTITUTO DI MEDICINA DEL LAVORO DELL'UNIVERSITA' DI MESSINA
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RICERCHE DI CITOCHIMICA EMATOLOGICA IN SOGGETTI ESPOSTI AL RISCHIO PROFESSIONALE D'INTOSSICAZIONE DA ACETONE E DA ACIDO ACETICO

ANGELO NUNZIANTE CESÀRO ANGELO GRANATA

Folia Medica 38: 957-66 (1955)

Sono state eseguite ricerche di citochimica ematologica su due gruppi di soggetti, facenti parte delle maestranze di un grande complesso industriale dell'Italia Settentrionale, dove si producono, tra l'altro, filati di acetato cellulosa.

Gli operai del primo gruppo erano esposti al rischio acetonico, quelli del secondo gruppo ad intossicazione da acido acetico; alcuni di essi avevano già subito, tempo addietro, qualche episodio di intossicazione subacuta, del resto ben superato e, dopo del quale, erano stati riammessi alle loro mansioni.

Nell'aria ambiente dei reparti di lavoro, come ad esempio nel reparto soluzioni per il primo gruppo e nel reparto acetilazione nel secondo, le concentrazioni di sostanze tossiche oscillavano come segue:

Acetone = mg/l 0,20-0,36 circa.

Acido acetico = mg/l 0,06-0,66 circa.

Il presente studio ha per obiettivo la descrizione degli esami di citochimica ematologica, eseguiti su strisci di sangue periferico, allo scopo di evidenziare le eventuali alterazioni, provocate dall'esposizione ai rischi professionali sopradetti.

METODI E RISULTATI.

Furono prelevati strisci di sangue periferico in soggetti a digiuno. Previa adeguata fissazione si procedette alle seguenti colorazioni:

1) panottica con i reattivi di May-Grünwald e Giemsa;
2) al sudan nero B. secondo Sheehan e Storey, per i lipidi;
3) all'acido periodico e reattivo di Schiff, secondo Hotchkiss, per i polisaccaridi;

4) dell'acido ribonucleico, col metodo di Unna e Pappenheim;
5) dell'acido desossiribonucleico, col metodo di Feulgen;
6) delle diastasi perossidasiche, col metodo di Sato;
7) delle diastasi perossidasiche, col metodo di Undritz;
8) delle diastasi ossidasiche, con la tecnica di Winckler e Schültze.

Si è determinato il coefficiente leucolipidico del sangue (CLS), per la cui metodica, così come per esaurienti particolari di tecnica si rimanda ad un precedente lavoro di Granata.

Analogamente sono stati introdotti due altri coefficienti: il leucossidasico (CLO) e il leucoperossidasico del sangue periferico (CLP). Per calcolare tali coefficienti si è dato, come già per il CLS, il fattore di proporzionalità 3 agli elementi cellulari presentanti una massima intensità reattiva, il fattore di proporzionalità 2 agli elementi di media reattività ed il fattore 1 agli elementi con un minimo di reazione. Il numero degli elementi cellulari si è moltiplicato per il fattore di proporzionalità relativo; i tre prodotti ottenuti si sono sommati e tale somma si è divisa per cento. Le formule dunque sono le seguenti:

$$\text{Coefficiente leucossidasico (CLO)} = \frac{3\,(Mx) + 2\,(Md) + 1\,(Mn)}{100}$$

$$\text{Coefficiente leucoperossidasico (CLP)} = \frac{3\,(Mx) + 2\,(Md) + 1\,(Mn)}{100}$$

Ad esempio: se si riscontrano all'esame di uno striscio di sangue su cento elementi 38 a reazione ossidasica massima, 44 a reazione ossidasica media e 18 a reazione ossidasica minima, si ha:

$$CLO = \frac{3\,(38) + 2\,(44) + 1\,(18)}{100} = \frac{114 + 88 + 18}{100} = 2{,}20$$

Teoricamente i coefficienti suddetti possono oscillare da un minimo di 1 (qualora tutti gli elementi abbiano una intensità di reazione minima) a un massimo di 3 (qualora tutti gli elementi esaminati presentino intensità massima).

L'introduzione di questi coefficienti è utile per un approssimativo giudizio quantitativo, ove non si possa praticare esame istospettrofotometrico.

Nei soggetti esaminati la durata di esposizione al rischio professionale variava da un minimo di 2 a un massimo di 25 anni per l'acetone e da un minimo di 7 a un massimo di 25 anni per l'acido acetico.

Si riportano i risultati sperimentali ottenuti:

1° Gruppo. — Soggetti esposti al rischio acetonico.

1°) Colorazione panottica al May-Grünwald-Giemsa. — Come è possibile vedere dalla tabella n. 1, sono stati osservati aumenti dei valori percentuali linfo-monocitari.

In sette casi su nove, la maggior parte dei monociti presentava sul nucleo granulazioni sparse di colorito azzurro cupo, quasi nero (fig. 1). Tutti i casi di monocitosi relativa possedevano tale reperto.

Non si sono notate forme anomale né della serie rossa, né di quella bianca. La formula di Arneth è risultata normale in tutti i casi.

TABELLA N. 1. — *Soggetti al rischio acetonico. Reperti sul sangue periferico. Colorazione panottica al May Grünwald-Giemsa.*

Casi		1°	2°	3°	4°	5°	6°	7°	8°	9°
nome		A B	M A	M F	C A	F P	D F	S A	R G	P G
età		55	47	52	57	35	60	36	47	34
anni di rischio		14	13	25	18	11	14	12	12	2
Formula leucocitaria	neutrofili	54	44	59	68	60	55	58	56	55
	eosinofili	2	2	—	—	2	2	—	—	2
	basofili	4	—	—	—	—	—	2	—	4
	linfociti	30	50	35	26	34	31	24	28	33
	monociti	10	4	6	6	4	12	16	16	6
granulazioni monocitarie		Pres.	ass.	ass.	pres.	pres.	pres.	pres.	pres.	pres.

2°) Colorazione al sudan nero B, secondo Sheehan e Storey, per i lipidi. — Dalla tabella n. 2 è rilevabile il comportamento dei granuli sudanofili leucocitari. Si ricorda che il valore normale del coefficiente leucolipidico del sangue (CLS) varia solitamente tra 2,53 e

2,81; per cui è evidente la diminuzione del patrimonio lipidico granulocitario in 7 dei nove casi esaminati.

TABELLA N. 2. — *Rischio acetonico; valutazione percentuale dei lipidi granulocitari e coefficienti leucolipidico.*

Casi		1°	2°	3°	4°	5°	6°	7°	8°	9°
Granulociti a reazione	massima	12	72	20	67	51	30	12	37	13
	media	59	20	61	21	38	56	53	45	60
	minima	29	8	18	12	11	14	30	18	67
Coeff. leuc. San. (CLS)		1,83	2,61	2,90	2,55	2,40	2,16	1,82	2,19	1,86

3°) Colorazione all'acido periodico e reattivo di Schiff, secondo Hotchkiss, per i polisaccaridi. — In tutti i casi ha dato risultato normale. Il citoplasma dei granulociti ha assunto una colorazione rosso-viola, la quale per intensità corrispondeva a quella assunta dagli stessi elementi dello striscio di sangue normale, preso come « test » della reazione.

4°) Colorazione dell'acido ribonucleico, secondo Unna e Pappenheim. — L'acido ribonucleico viene evidenziato nel nucleo e nel citoplasma degli elementi della serie bianca. L'intensità della reazione ha dato i seguenti risultati: *normale* per i casi 1, 2, 3, 6; — *mediocre* nel caso 8; — *debole* nei casi 4 e 9; — *molto debole* nei casi 5 e 7.

5°) Colorazione dell'acido desossiribonucleico, con il metodo di Feulgen. — Nella totalità dei casi i nuclei degli elementi leucocitari sono apparsi con una intensità cromatica diminuita rispetto al « test » normale.

6°) Colorazione delle diastasi perossidasiche, con il metodo di Sato e Sekjia. — Dalla tabella n. 3 si desume che il comportamento delle diastasi perossidasiche è pressocchè normale, giudizio che viene confermato dal coefficiente leucoperossidasico (CLP), il quale in soggetti normali varia da 2 a 2,50.

TABELLA N. 3. — *Rischio acetonico; valutazione percentuale delle diastasi perossidasiche e coefficiente leucoperossidasico. Colorazione di Sato.*

Casi		1°	2°	3°	4°	5°	6°	7°	8°	9°
Granulociti a reazione	massima	35	35	40	49	36	63	52	37	28
	media	42	56	43	47	46	27	40	52	52
	minima	23	9	17	4	18	10	8	11	20
Coef. Leuco-Perisid. (CLP)		2,12	2,26	2,23	2,45	2,18	2,03	2,44	2,26	2,08

7°) *Colorazione delle diastasi perossidasiche con il metodo di Undritz.* — Merita un particolare accenno la presenza, sullo sfondo aureo del citoplasma leucocitario, di granulazioni intesamente colorate dal marrone scuro al nerastro, particolarmente copiose in certi neutrofili, assenti invece negli cosinofili. I monociti si presentano spesso con tali grosse granulazioni, senza alcun sfondo che ricordi la tipica reazione di Undritz. Un esempio tipico di granulocita neutrofilo con granulazioni nerastre è riprodotto nella fig. 2.

In genere la colorazione in esame mostra una riduzione del patrimonio diastasico perossidasico, evidenziato nel citoplasma dei leucociti. L'apparente contrasto con il risultato, ottenuto mediante il metodo di Sato, dimostra ancora una volta la maggior finezza e specificità della reazione di Graham-Knoll-Undritz.

Riassumendo si è trovato un tenore perossidasico normale con mancanza di granulazioni nerastre nei casi 8 e 9; un tenore perossidasico discreto, con presenza di granulazioni nerastre nei casi 1, 2, 4, 5, 6: una intensità debolissima con numerosissime granulazioni anomale, coprenti talvolta i due terzi del protoplasma, nel caso 7.

8°) *Colorazione delle diastasi ossidasiche, con la tecnica di Winckler e Schültze.* — L'esame della tabella n. 4 dimostra una diminuzione delle ossidasi leucocitarie in sei casi su nove. Ciò si desume facilmente dal coefficiente leucossidasico (CLO) che nel normale deve oscillare tra 2 25 e 2,75.

TABELLA N. 4. — *Rischio acetonico. Valutazione percentuale delle diastasi ossidasiche e coefficiente leucossidasico. Colorazione di Winckler e Schültze.*

Casi		1°	2°	3°	4°	5°	6°	7°	8°	9°
Granulociti a reazione	massima	38	16	31	41	23	68	35	30	54
	media	44	56	45	46	50	24	40	46	37
	minima	18	28	24	13	27	8	25	24	9
Coef Leuco-Ossid. (CLO)		2,20	1,88	2,07	2,28	1,96	2,60	2,10	2,06	2,45

2° Gruppo. — Soggetti esposti al rischio d'intossicazione da acido acetico.

1°) *Colorazione panottica al May Grünwald-Giemsa.* — Linfocitosi relativa è stata osservata in cinque casi su nove e in quattro casi anche monocitosi. Le granulazioni a colorazione intensa e nerastra simili a quelle osservate nell'intossicazione da acetone sono apparse in cinque casi. Abbastanza frequente il reperto di granulociti basofili, come è possibile vedere nella tabella n. 5, nella quale sono altresì esposti anche gli anni di età e di rischio professionale dei soggetti esaminati.

TABELLA 5. — *Rischio d'intossicazione da acido acetico. Reperti su strisci di sangue periferico. Colorazione al May Grünwald-Giemsa.*

Casi		1°	2°	3°	4°	5°	6°	7°	8°	9°
nome		R O	P Q	P A	A E	M O	D G	P P	B G	B L
età		39	34	47	47	38	37	46	55	45
anni di rischio		7	10	24	9	9	9	9	25	10
formula leucoritaria	neutrofili	68	61	52	58	58	55	67	68	53
	cosinofili	2	2	—	2	2	3	2	2	2
	basofili	1	2	—	2	—	1	1	—	2
	linfociti	22	31	38	30	24	31	26	24	33
	monociti	7	4	10	8	16	10	8	6	10
granul. monocitarie		ass.	pres.	pres.	pres.	pres.	ass.	pres.	ass.	ass.
formula di Arneth		N	N	N	N	N	→	→	N	→

2°) *Colorazione al sudan nero B, secondo Sheehan e Storey, per i lipidi.* — Un quadro analogo a quello osservato con il rischio acetonico, è visibile nella tabella n. 6. Dai valori del coefficiente leucolipidico del sangue (CLS) si desume una riduzione del patrimonio sudanofilo dei granulociti in sette casi su nove.

I valori del CLS non raggiungono però in nessun caso i minimi del rischio acetonico, che erano di 1,82-1,83.

TABELLA N. 6. — *Rischio d'intossicazione da acido acetico. Valutazione percentuale dei lipidi granulocitari e coefficiente leucolipidico del sangue.*

Casi		1°	2°	3°	4°	5°	6°	7°	8°	9°
Granulociti a reazione	massima	78	12	47	15	62	46	14	55	30
	media	16	78	40	67	36	48	71	35	54
	minima	6	10	13	18	2	6	15	10	16
CLS		2,72	2,02	2,34	1,97	2,60	2,40	1,99	2.45	2,14

3°) *Colorazione all'acido periodico e reattivo di Schiff, secondo Hotchkiss, per i polisaccaridi.* — Nei casi esaminati ha dato intensità di reazione corrispondente a quella del « test » normale.

4°) *Colorazione dell'acido ribonucleico, con il metodo di Unna e Pappenheim.* — Per quanto il reperto sia positivo, sia per il nucleo che per il protoplasma delle cellule leucocitarie, tuttavia l'intensità della colorazione nucleare è molto meglio evidenziata. Ciò costituisce un dato di normalità che non si era osservato nei casi di rischio acetonico. Il grado di colorazione può essere così sintetizzato:

normale nei casi 2, 4, 7 — *debole* nei casi 1, 3, 6 — *molto debole* nei casi 5, 8, 9.

5°) *Colorazione dell'acido desossiribonucleico, con il metodo di Feulgen.* — Come per il rischio acetonico, anche qui la totalità dei casi presenta una diminuzione di lieve entità della specifica colorazione nucleare.

6°) *Colorazione delle diastasi perossidasiche, con il metodo di Sato e Sekjia.* — La tabella n. 7 mostra il comportamento delle diastasi perossidasiche dei nove casi in esame. Anche qui, come nel rischio acetonico, il coefficiente leucoperossidasico è risultato nei limiti della normalità.

TABELLA N. 7. — *Rischio d'intossicazione da acido acetico. Valutazione percentuale delle diastasi perossidasiche e coefficiente leucoperossidasico. Colorazione di Sato.*

Casi		1°	2°	3°	4°	5°	6°	7°	8°	9°
Granulociti a reazione	massima	39	60	30	60	40	54	31	29	27
	media	49	29	56	24	48	35	52	58	61
	minima	12	11	14	16	12	11	17	13	12
CLP		2,27	2,49	2,16	2,44	2,28	2,43	2,14	2,16	2,15

7°) *Colorazione delle diastasi perossidasiche con il metodo di Undritz.* — Le granulazioni nerastre sono presenti anche qui come nel rischio acetonico. Il tenore perossidasico è in genere basso e può essere così riassunto: *normale* nei casi 2, 3 e 6; *debole* nei casi 1, 4, 5, 7, 8 e 9.

In alcuni casi (5 e 9) i monociti si presentano con grosse granulazioni nerastre e privi di sfondo citoplasmatico.

8°) *Colorazione delle diastasi ossidasiche, con la tecnica di Winckler e Schültze.* — In cinque casi su nove le ossidasi leucocitarie risultano diminuite e in un caso abbastanza sensibilmente tanto da abbassare il coefficiente leucossidasico a 1,72 (minimo del rischio acetonico 1.88).

Vedasi per maggiori particolari la tabella n. 8.

TABELLA N. 8. — *Rischio d'intossicazione da acido acetico. Valutazione delle diastasi ossidasiche e coefficiente leucossidasico.*

Casi		1°	2°	3°	4°	5°	6°	7°	8°	9°
Granulociti a reazione	massima	28	60	58	35	50	26	42	44	13
	media	54	36	34	43	34	20	40	39	57
	minima	18	4	8	22	16	54	18	17	30
CLO		2,10	2,56	2,50	2,13	2,34	1,72	2,24	2,27	1,83

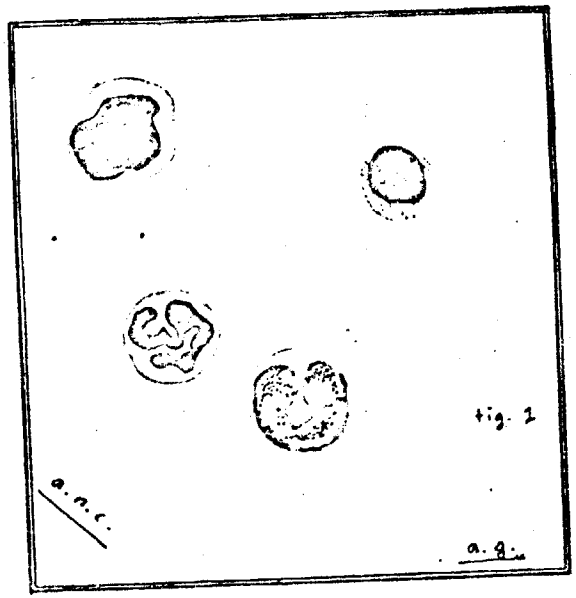

Fig. 1. — Rischio acetonico. — Colorazione panottica di Pappenheim. — A destra, monocita con granulazioni anomali nucleari intensamente basofile.

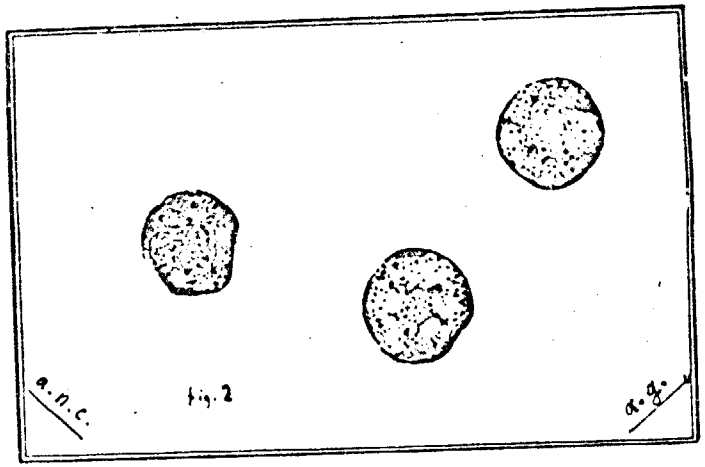

Fig. 2. — Rischio acetonico. — Colorazione di Graham-Knoll-Undritz. — Granulociti neutrofili a debole tenore perossidasico. — In due elementi sono visibili le granulazioni nerastre descritte nel testo.

Considerazioni e conclusioni

a) Nel gruppo di operai esposti al rischio acetonico si sono riscontrate le seguenti alterazioni dalla norma: frequente monocitosi e linfocitosi relativa, frequente diminuzione della sudanofilia con relativo abbassamento del coefficiente leucolipidico del sangue; indebolimento pressocchè generale delle ribo- e delle desossiribonucleine leucocitarie (reazioni di Unna-Pappenheim e di Feulgen); normalità invece per quanto riguarda i polisaccaridi e le perossidasi evidenziate col metodo di Sato; in buona parte dei casi le ossidasi e le perossidasi, quest'ultime evidenziate col metodo di Undritz, mostrano una diminuzione più o meno evidente;

b) nel gruppo di operai esposti alla intossicazione professionale da acido acetico si sono dimostrate alterazioni che si avvicinano nella maggior parte dei casi a quelle del gruppo precedente, si ha però l'impressione che esse siano minori come intensità e insorgano dopo un maggior tempo di esposizione al rischio. Anche in questo gruppo esiste in gran parte dei casi monocitosi e linfocitosi relativa, diminuzione della sudanofilia, indebolimento del patrimonio ribo- e desossiribonucleico, diminuzione delle ossi-perossidasi. Normali invece i polisaccaridi e le perossidasi con il metodo di Sato;

c) in ambedue i gruppi si è constatata la presenza di granulazioni fortemente colorate e basofile sul nucleo di alcuni monociti. Tali granulazioni sono state evidenziate solo mediante il metodo della colorazione panottica e sono rimaste assolutamente incolori con gli altri metodi di colorazione usati;

d) la reazione di Undritz oltre a dimostrare una diminuzione del contenuto perossidasico citoplasmatico nei granulociti ha posto in evidenza spesso presenza di granulazioni azzurro scure, disperse nell'ambito del protoplasma fino a ricoprirlo talvolta in buona parte. Reperti così anormali sono stati evidenziati in ambedue i gruppi esaminati.

RIASSUNTO

Sono state praticate ricerche di citochimica ematologica su due gruppi di soggetti, gli uni esposti al rischio professionale di intossicazione da acetone, gli altri esposti a quello da acido acetico.

Oltre a una monocitosi e linfocitosi relativa, evidenziate mediante la colora-

zione panottica, si sono potute rilevare, con le recenti metodiche di emocitochimica, alterazioni al patrimonio lipidico, nucleoproteico e ossidiastasico dei leucociti.

RESUME

Des recherches de cytochimie hématologique ont été effectuées sur deux catégories de sujets: la première exposée au danger professionnel d'intoxication par acétone, la deuxième exposée à celui de l'acide acétique.

On a trouvé, avec la coloration panoptique, une lymphocytose et une monocytose relatives et, avec les modernes méthodes hémocytochimiques, une altération du patrimoine leucocytaire des lipides, des acides nucléiques et des oxy-peroxydases.

SUMMARY

Haematologic cytochemical tests were carried out with two group of workers, of which one group were exposed to professional risk of acetone intoxication and the other to that of acetic acid.

In addition to monocytosis and relative lymphocytosis being made evident by panoptical colouring, modifications in the lipid nucleoproteinic and ossidyastatic depot in the leukocytes were found.

BIBLIOGRAFIA

GRANATA A. — Ricerche sui lipidi dei granulociti nella silico-tubercolosi. Folia Medica, 38: 26-36 (1955).

NUNZIANTE CESÀRO A. e GRANATA A. — Ricerche di citochimica ematologica in talune tecnopatie disemizzanti. Folia Medica, 36: 861-880 (1953).

UNDRITZ E. — Eine Modifikation der Peroxydase-reaktion nach Knoll zu Bestimmung der Monozyten (Verhandl.-Schw. Naturforsch. Ges. 121: 20 (1941).

Rat Strain Differences in the Utilization of Glucose-U-^{14}C and Acetate-1-^{14}C for Fat Synthesis (35980)

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In our laboratories the fat metabolism of BHE and Wistar rats has been extensively investigated (1-3). It has been shown that the BHE rats tend to accumulate more fat in the liver than do Wistar rats under comparable dietary treatment. Recently efforts have been made to study the mechanisms controlling lipogenesis in these two strains of rats. The activity of liver glucose-6-phosphate dehydrogenase (G6PD) was found to be significantly higher in BHE than Wistar (4, 5). A higher level of G6PD could be taken as a sign of a higher hexosemonophosphate (HMP) shunt activity, and it was associated with higher liver lipids in BHE rats (3, 4). A more direct indication of shunt activity was obtained by measuring the respiratory ratio of expired $^{14}CO_2$ from glucose-1-^{14}C vs glucose-6-^{14}C. This ratio proved to be higher for the Wistar strain than for BHE rats (4). It appeared that, in spite of low G6PD activity in liver, Wistar rats utilized the shunt pathway in some tissues to a greater extent than did BHE animals.

To further clarify strain differences in fat metabolism, the present study involving the metabolic fates of glucose-1-^{14}C (G-1-^{14}C), glucose-6-^{14}C (G-6-^{14}C), uniformly labeled glucose (G-U-^{14}C) and acetate-1-^{14}C was undertaken. The incorporation of label from G-U-^{14}C and acetate-1-^{14}C in the lipid of various portions of the body and lipid classes of plasma and liver was investigated. The respiratory ratio of $^{14}CO_2$ from G-1-^{14}C vs G-6-^{14}C was reexamined, since the diet used in the present study did not contain cholesterol, as had the previous diet (4).

Materials and Methods. Three groups of male weanling BHE and Wistar rats were housed individually in wire bottom cages and fed a diet consisting of the following (g/100 g): casein, 27; methionine, 0.2; beef tallow, 14; corn oil, 2; salt mix, 4; cellufloor, 2; vitamin mix, 1; and glucose, 49.8. The detailed composition of salt mix and vitamin mix and the care of rats were as previously reported (4). All rats were fed this diet for 70 days (to an age of 3 months), after which they were fasted overnight. At 8 a.m. each rat was then refed 4 g of diet. One hour after the start of refeeding, five BHE and five Wistar each were given intraperitoneally, 2 μCi of G-1-^{14}C[1] in 0.5 ml of isotonic saline solution containing 5 mg of nonlabeled dextrose and each rat was immediately placed in an individual all-glass metabolic cage. The respiratory $^{14}CO_2$ was trapped during each of the first four 1-hr periods in a 3:7 mixture of ethanolamine and ethylene glycol monoethyl ether. Radioactive $^{14}CO_2$ was assayed by liquid scintillation counting (4). After the last sample was collected, all rats were transferred to their regular cages and were maintained on the diet for 3 days. The same rats were then fasted overnight, refed 4 g of diet, and injected with 2 μCi of G-6-^{14}C.[1] The ratio between respiratory 1-$^{14}CO_2$ and 6-$^{14}CO_2$ was determined for each rat.

The second group of BHE and Wistar rats was given 4 μCi of G-U-^{14}C[1] each. After 4 hr each rat was killed by decapitation. Blood was collected in heparinized tubes and plasma was separated by centrifugation. Liver, abdominal fat pads, and carcass[2] were kept frozen for chemical analysis later.

[1] Specific activity of glucose-1-^{14}C, 15.9 μCi/mg; glucose-6-^{14}C, 21.6 μCi/mg; glucose-U-^{14}C, 21.6 μCi/mg, acetate-1-^{14}C, 354 μCi/mg. All radioactive compounds were purchased from Nuclear Chicago.

[2] Eviscerated carcass.

Each of the third group of rats was given 2 μCi of acetate-1-^{14}C[1] in 0.5 ml of isotonic saline. After 4 hr, each rat was also sacrificed in the same manner as was in the previous group. Blood was collected and liver, abdominal fat pads, and carcass[2] were kept frozen for chemical analysis later.

Analysis of tissue lipids. Liver. Total liver lipid was extracted with 2:1 (v/v) chloroform:methanol and purified by washing 5–7 times with 0.04% $CaCl_2$ (6). The lipid extract was evaporated to dryness and weighed. A known portion of lipid was used for measuring total radioactivity by liquid scintillation counting.

Fractionation of liver lipids. From another known portion of liver lipid, phospholipids were separated from the neutral lipids by a silicic acid column (7, 8). After drying the separated fractions, neutral lipids and phospholipids were weighed. A portion of phospholipids was used for measuring radioactivity by liquid scintillation counting, and the neutral fat was further separated into fatty acid, glyceride-glycerol, and cholesterol after saponification with alcoholic KOH (9). Cholesterol was determined by the method of Kabara (9) and glycerol by the Carlson procedure (10). The radioactivity in a known amount of cholesterol and glycerol was determined (9). Fatty acids were extracted with *N*-hexane after acidifying the saponified mixture to pH 2–3 following digitonide precipitation of cholesterol (11). The total radioactivity of fatty acids was counted although their quantity was not determined.

Lipids in plasma. An equal volume (usually 1.5 ml) of plasma from each rat was pooled according to strain and isotopic treatment. After a thorough mixing, duplicate 0.05 ml aliquots were taken for determination of total cholesterol. Four samples of 1.0 ml aliquot were used for extraction of total lipids (12). The extract was washed 5–7 times with 0.04% $CaCl_2$. Two lipid samples were used for determining total radioactivity, and the other two samples were used for further fractionation. The separation of phospholipids from neutral lipids was also accomplished by silicic acid column chromatography. The quantity of phospholipids was determined by a colorimetric method (13) and radioactivity was determined by liquid scintillation counting. The neutral lipid fraction was saponified with 2.5 *N* alcoholic KOH. The same method as for the liver lipids was used for quantitative measurement of cholesterol and glycerol. Cholesterol and fatty acid were also separated by thin-layer chromatography with the running solvent 80:20:1 (v/v) *N*-hexane:ethyl ether:acetic acid. A mixture of known reference compounds was applied on the same plate as a guide. Radioactivity was then determined in the isolated cholesterol and fatty acids.

Abdominal fat pads. Epididymal and perirenal fat pads were quickly excised and weighed. The respective fat pads were minced and duplicate samples (200–300 mg) were used for fat extraction (14) and measurement of radioactivity.

Carcass fat. Carcass fat was extracted as previously described (15). A known amount of fat was used for counting the radioactivity.

Results. Figure 1 shows quantity of $^{14}CO_2$ collected over each of four successive 1-hr periods from BHE and Wistar rats given G-1-^{14}C and G-6-^{14}C. The 4-hr collections of CO_2 showed a ratio of $^{14}CO_2$ from injected glucose-1-^{14}C vs glucose-6-^{14}C, which was 0.93 in BHE animals and 1.14 in Wistar rats. The difference in this ratio was consistent but small and may not have been meaningful.

Lipid distribution within the bodies of BHE vs Wistar rats is presented in Table I. Present data, indicating that BHE rats had larger perirenal fat pads and more fat in liver and plasma than did Wistar rats, confirmed previous findings (2, 5).

The higher content of fat in the livers and plasma of BHE rats was accompanied by a greater incorporation in lipids of radioactivity from acetate-1-^{14}C. However, label from G-U-^{14}C was higher only in the liver fat of BHE rats. The BHE animals showed a greater incorporation of acetate-1-^{14}C but not of G-U-^{14}C in the perirenal fat than did the Wistar rats (Table II).

The level of fractionated lipids of liver and plasma of BHE vs Wistar rats is shown in

TABLE I. Body Lipid Distribution in BHE and Wistar Rats.

Strain	Carcass Total	Carcass (%)*	Fat pads (g) Epididymal	Fat pads (g) Perirenal	Liver Total (mg)	Liver (%)	Plasma (mg/100 ml)
BHE (9)	40.8 ± 2.8	13.8 ± 0.96	7.1 ± 0.54	9.2 ± 0.76	775 ± 30	7.0 ± 0.21	525
Wistar (9)	40.4 ± 1.7	13.3 ± 0.64	8.5 ± 0.55	8.7 ± 0.63	569 ± 38.9	5.3 ± 0.34	477

* One gram of fat per 100 g of wet weight of carcass.
† Mean ± SE.
‡ $p \leq .01$.

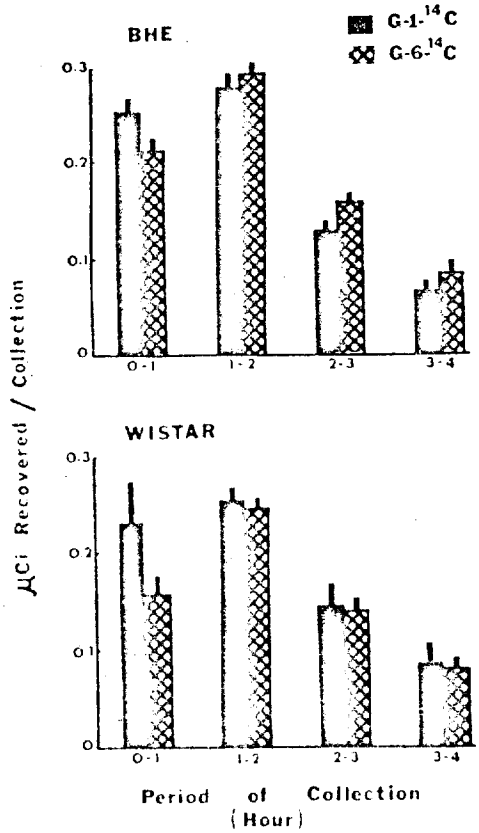

FIG. 1. Recovery of $^{14}CO_2$ from rats after the administration of glucose-1-^{14}C and glucose-6-^{14}C: five rats per group.

Table III. The amount of radioactivity and its relative percentage distribution in different fractions from the total is shown in Table IV. The level of liver phospholipids, glycerides, and cholesterol were significantly higher in BHE than in Wistar rats, and the amount of radioactivity incorporated into these fractions from acetate-1-^{14}C was also markedly higher in BHE than in Wistar rats. The level of fatty acids in Plasma neutral lipids was not determined but was found to contain a higher amount of radioactivity from acetate-1-^{14}C in BHE than in Wistar rats.

The relative percentage distribution of total incorporated radioactivity to lipid classes in liver and plasma lipids was different between strains. The pattern of distribution be-

TABLE II. Incorporation of Label from Glucose-U-^{14}C and Acetate-1-^{14}C into Body Lipids of BHE and Wistar Rats.

Strain	Recovery of radioactivity (dpm/μCi injected)				
	Carcass	Epididymal	Perirenal	Liver	Plasma[a] per ml
	After injection of glucose-U-^{14}C				
BHE (5)[b]	62,151[c] ±3521	1705 ±252	2576 ±129	8568[e] ±867	30.85
Wistar (5)	63,239 ±7242	3925 ±965	3409 ±549	4815 ±180	44.61
	After injection of acetate-1-^{14}C				
BHE (4)	133,449 ±14,522	17,876 ±8337	18,283[e] ±6345	31,936[d] ±7861	171.7
Wistar (4)	207,497 ±69,534	3672 ±3260	1250 ±218	8016 ±883	50.5

[a] Pooled plasma sample used.
[b] Number of rats is given in parentheses.
[c] Mean ± SE.
[d] $p \leq .05$.
[e] $p \leq .01$.

tween liver and plasma was not similar. This discrepancy might be due to the transport of lipids in plasma from various origins rather than from liver alone. Percentage of radioactivity in phospholipids from total compared with that in neutral lipids in liver was higher in Wistar than in BHE rats, but BHE had higher percentage in glyceride glycerol from G-U-^{14}C and higher percentage in fatty acids and cholesterol from acetate-1-^{14}C.

Discussion. The ratio of 1-^{14}C 6-^{14}C of $^{14}CO_2$ from administered G-1-^{14}C and G-6-^{14}C may serve as an indicator for the body as a whole but does not indicate the contribution of tissues or organs to the total HMP activity. Lee and Lucia (16) reported that animals restricted in caloric intake showed little whole-body HMP activity, as indicated by a 1-CO_2/6-CO_2 value close to 1.0, yet showed an elevated activity of hepatic G6PD. They interpreted that an increased utilization of the HMP shunt in the liver may be masked by the activity of Embden-Meyerhof pathway in other tissues. The present study presents a similar situation. Data from whole-body radiorespirometry indicates that BHE animals show less HMP activity than animals of the Wistar strain;

TABLE III. Phospholipid, Glyceride, and Cholesterol Levels in Liver and Plasma[a] of BHE and Wistar Rats.

Strain	Phospholipids (mg)	Glyceride (μmoles)	Cholesterol (mg)
	Liver		
BHE (9)[b]	366 ± 30[c,d]	157 ± 26[e]	28 ± 2[d]
Wistar (9)	297 ± 17	82 ± 10	20 ± 2
	Plasma (per 100 ml)		
BHE	154	5.0	70.2
Wistar	159	5.8	64.7

[a] Pool of plasma sample was used.
[b] Number in parentheses represents number of rats.
[c] Mean ± SE.
[d] $p \leq .05$.
[e] $p \leq .01$.

TABLE IV. Distribution of Radioactivity in Phospholipids, and Glycerol, Fatty Acids, and Cholesterol of Neutral Lipids of Liver and Plasma in BHE and Wistar Rats.

			Neutral lipids					
	Phospholipids		Glycerol		Fatty acids		Cholesterol	
Strain	Total (dpm)	(%)[a]	Total (dpm)	(%)	Total (dpm)	(%)	Total (dpm)	(%)
			After injection of glucose-U-^{14}C (per μCi injected)					
			Liver					
BHE (5)[b]	4739		3108[c]		163		Nil	0
	±509[c]	57	±581	41	±69	2		
Wistar (5)	3160		888		119		Nil	0
	±259	77.6	±165	19.7	±16	2.7		
			Plasma (per ml)					
BHE	14	39	20	56	1.7	5	Nil	0
Wistar	13	29	30	66	2.4	5	Nil	0
			After injection of acetate-1-^{14}C (per μCi injected)					
			Liver					
BHE (4)	11,211[d]		561		12,716[d]		4810[d]	
	±3196	44.3	±149	1.1	±5519	39.6	±1478	15
Wistar (4)	4810				2374		358	
	±771	63.8	Nil		±271	31.5	±59	4.7
			Plasma (per ml)					
BHE	26	22	21	18	55	47	15	13
Wistar	7	15	19	40	13	28	8	17

[a] Relative percentage of total radioactivity recovered from liver phospholipids and neutral lipids.
[b] Number of rats is given in parentheses.
[c] Mean ± SE.
[d] $p < .05$.
[e] $p < .01$.

yet the BHE have been shown to have higher levels of hepatic G6PD under a variety of dietary conditions (4, 5), including the feeding of the same diet used in this study (5).

Results from a time course study suggested that labeled fat in adipose tissue and liver up to 6 hr after administered labeled acetate to fed animals represented the synthesis in these tissues (17). Since the present results were obtained from fed animals 4 hr after the administration of labeled compounds, isotope incorporation into fat in the tissues has been assumed to represent synthesis in these tissues. The markedly higher amount of radioactivity from G-U-^{14}C acetate 1-^{14}C was found in liver fat of BHE than that of Wistar rats (Table II) and indicates the BHE rats had a higher rate of hepatic synthesis of fat. This may explain the higher level of liver fat in the present study (Table I), as well as the previous finding (5) for BHE rats over Wistar rats. Because of wide variations between individuals, the differences in amount of radioactivity in lipids of other tissues (indicated as high SE), were not significant except for a greater incorporation of label from acetate-1-^{14}C into lipids of perirenal fat of BHE rats. Thus, the rate of fat synthesis in tissues, especially in liver, shows strain differences at this age.

Most of the glucose label incorporated into the neutral lipids of liver was found in the glycerol moiety. Apparently, an appreciable portion of glucose in the liver, on being converted to dihydroxyacetone phosphate during glycolysis, was reduced to α-glycerophosphate, the chief glycerol precursor of triglyceride in the liver. A greater portion of the glucose label appeared in the glycerol portion of neutral lipids in BHE than Wistar animals. A possible explanation is a higher activity of α-glycerophosphate dehydrogenase in

the livers of BHE than Wistar rats.[3]

In the present studies, BHE rats had a higher rate of synthesis of glyceride-glycerol in liver but also of fatty acids and cholesterol (Table IV). Livers of BHE animals synthesized phospholipid from acetate-1-^{14}C faster than did the Wistar animals. However, the strain difference in lipogenesis was most prominent in the neutral lipid fraction, which was larger and incorporated more acetate label in BHE than Wistar animals. Since it was known that hepatic glucose-6-phosphate dehydrogenase (4, 5) and malic enzyme (18) were markedly higher in BHE than in Wistar, the higher rate of fatty acid synthesis from acetate could be the result of the activity of the enzyme systems which produced NADPH, a factor required for fatty acid synthesis.

The forms of lipogenesis observed here included synthesis of fatty acids, glyceride-glycerol, phospholipids, and cholesterol. The results suggest that the major difference in fat metabolism between these two strains occurred in liver, and BHE had a higher rate of lipogenesis in this organ.

Summary. The difference in utilization of glucose and acetate for fat synthesis between rats of the BHE and Wistar strains was studied after the administering of acetate-1-^{14}C or -^{14}C-glucose labeled either uniformly or at the C-1 or C-6 position. Wistar rats were found to show a higher 1-$^{14}CO_2$/6-$^{14}CO_2$ ratio than BHE animals; but the difference was too small to demonstrate a real difference in whole-body utilization of the HMP shunt. The BHE animals incorporated a larger fraction of glucose and acetate label into the liver lipids than did Wistar animals; but incorporation of these labels into total carcass lipid was similar in the two strains at this age. Results indicated that hereditary differences in lipogenesis between these strains occur chiefly in the liver, where BHE animals synthesize fatty acids, glyceride-glycerol, phospholipids, and cholesterol at a higher rate than Wistar animals.

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1. Marshall, M. W., and Hildebrand, H. E., J. Nutr. 79, 227 (1963).

2. Taylor, D. D., Conway, E. S., Schuster, E. M., and Adams, M., J. Nutr. 91, 375 (1967).

3. Chang, M. L. W., and Adams, M., Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24, 220 (1965).

4. Chang, M. L. W., Schuster, E. M., Lee, J. A., Spodarass, C., and Benton, D. A., J. Nutr. 96, 368 (1968).

5. Chang, M. L. W., Lee, J. A., Schuster, E. M., and Trout, D. L., J. Nutr. 101, 323 (1971).

6. Folch, J., Lees, M., and Sloane Stanley, G. H., J. Biol. Chem. 226, 497 (1957).

7. Galanos, D. S., and Kapoulas, V. M., Biochim. Biophys. Acta 98, 278 (1965).

8. Bergstrom, B., Acta Physiol. Scand. 25, 101 (1952).

9. Kabara, J., J. Lab. Clin. Med. 50, 146 (1957).

10. Carlson, L. A., J. Atheroscler. Res. 3, 334 (1963).

11. Nugteren, D. H., Biochim. Biophys. Acta 106, 280 (1965).

12. Caraway, W. T., "Microchemical Methods for Blood Analyses," p. 82. Thomas, Springfield, IL (1960).

13. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L., and Farr, A. L., J. Biol. Chem. 207, 1 (1954).

14. Colowick, S. P., and Kaplan, N. O., Methods Enzymol. 3, 299 (1957).

15. Ass. Offic. Agr. Chem., "Official Methods of Analysis," p. 17. Washington, DC (1970).

16. Lee, M., and Lucia, S. P., J. Nutr. 74, 249 (1961).

17. Leveille, G. A., Proc. Soc. Exp. Biol. Med. 125, 85 (1967).

18. Berdanier, C. D., Szepesi, B., Moser, P., and Dietchenko, S., Proc. Soc. Exp. Biol. Med. 137, 663 (1971).

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[3] Unpublished data.

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TABLE XII. ORGANIC DERIVATIVES OF CARBOXYLIC ACIDS

a) Liquids (b) Listed in order of increasing atmospheric b.p.)*

No	Name	Boiling point, °C	Melting point, °C	n_D^{20}	D_4^{20}	p-Toluidide	Anilide	p-Bromophenacyl ester	Amide	Methyl ester	Ethyl ester	Miscellaneous
1	Thioacetic acid	93	.....	.....	1.074[20]	130	76	.....	108	.....	.....	.....
✓ 2	Formic acid	100.7	8.4	1.37143	1.22026	53	50	140; 135	.....	.....	.....	p-Nitrobenzyl ester, 31
✓ 3	Acetic acid (Ethanoic acid)	118.2	16.6	1.36976; 1.3721	1.04926	153; 147	114	86.0	82	.....	.....	p-Nitrobenzyl ester, 78
4	Difluoroacetic acid	134.5	.....	.....	.....	.....	.....	.....	52	.....	.....	.....
5	Acrylic acid	141; 140	13	1.4224	1.06212[20]	141	104-5, w.	.....	84.5, pet. eth.	.....	.....	.....
6	Propionic acid (Propanoic acid)	141	−20.8	1.3868	0.99336	126; 123	106	63.4	81	.....	.....	p-Nitrobenzyl ester, 31
7	Propiolic acid	144d.	18	.....	1.1391[15]	.....	87	.....	61-2	.....	.....	.....
8	Isobutyric acid (Isobutanoic acid)	154.7	−46.1	1.3920	0.94791	108.5-9.5	105	76.8	128; 129	.....	.....	.....
9	Methacrylic acid	161	16	1.429	1.015	.....	.....	.....	102-6	.....	.....	p-Bromoanilide, 116
10	n-Butyric acid (n-Butanoic acid)	162.2; 164	−5.5; −8	1.3983; 1.3979	0.95790	75	96; 97	63	115.6	.....	.....	p-Nitrobenzyl ester, 35
11	Pyruvic acid (α-Oxopropionic acid)	165d.; 80[15]	13.6	1.4138	1.26681[15]	109; 130	104, subl.	.....	124-5; 145	.....	.....	2,4-Dinitrophenylhydrazone, 218, yel., al.
12	Vinylacetic acid (3-Butenoic acid)	169, 163	−35	1.4221	1.0094	.....	58	.....	73	.....	.....	.....
13	Isocrotonic acid (cis-(d)-Crotonic acid, cis-2-Butenoic acid)	169	15	1.4456	1.0265	132	101-2	81	101.2	.....	.....	.....
14	d,l-2-Methylbutanoic acid (Ethylmethylacetic acid)	176.7; 174	.....	1.4052	0.938[20]	92.5; 3.0	110	55	112	.....	.....	.....
15	Isovaleric acid (3-Methylbutanoic acid)	176.5	−30.0	1.4043	0.92623	106.7	109.5 (cor.)	68.0	135; 137	.....	.....	.....
16	n-Amylpropiolic acid (1-Heptyne-1-carboxylic acid)	180; 220d.	f.p.: 2.5	.....	.....	68, bz	.....	.....	91	.....	.....	Nitrile, b.p.: 194-6; o-Toluidide, 60, pet. eth.
17	3,3-Dimethylbutanoic acid (tert-Butylacetic acid)	184; 96[10]	6.7	1.4096	0.9124	134	132, et ac.-pet. eth	.....	132	b.p.: 126	.....	.....
18	d,l-α-Chloropropionic acid	186	.....	.....	.....	124	92	.....	80	.....	.....	.....
19	Cyclopropanecarboxylic acid	186; 182.4	17; 18.9	1.43901	1.0885	.....	.....	.....	125	.....	b.p.: 134; n_D^{20}: 1.41902; D_4^{20}: 0.96078	.....
20	n-Pentanoic acid (n-Valeric acid)	186.4	−34.5	1.4086	0.93922	74	63	75	106	.....	.....	.....
21	2,2-Dimethylbutanoic acid (Dimethylethylacetic acid)	187; 190	−15.0	1.4141; 1.4145	0.9276	83.0-.5	92, 90.1	.....	103	.....	.....	p-Phenylphenacyl ester, 86
22	Allylacetic acid (4-Pentenoic acid)	188.9	.....	1.4341; 1.4283	0.98431[20]	.....	.....	.....	94, b.p.: 230	.....	b.p.: 144.6	.....
23	Cyclopropylacetic acid	190[750]	.....	1.43207	.....	.....	.....	.....	.....	.....	.....	p-Phenylphenacyl ester, 83
24	d,l-2,3-Dimethylbutanoic acid (Isopropylmethylacetic acid)	191.7	−1.5	1.4146	0.9275	112.6	78.4	.....	132	.....	.....	p-Phenylphenacyl ester, 74
25	Dichloroacetic acid	194	5.6	1.4659	1.5634	153	118	99	98, subl.	.....	.....	.....

*Derivative data given in order: m.p., crystal color, solvent from which crystallized.

EFFECT OF FRENCH DRESSINGS, VINEGARS, AND ACETIC ACID ON RATE OF LOSS OF VITAMIN C IN RAW CABBAGE

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In 1936 some experiments with pure vitamin C [1] and vinegars were carried out by the senior author in an attempt to secure information which might help to explain the vitamin C losses which occur in vegetables during pickle making. In the first experiment a comparison was made of vitamin C losses in mixtures of a solution of pure ascorbic acid (in distilled water) with distilled water, cider vinegar (raw and boiled), and five per cent acetic acid. The results (Table 1) show that the immediate loss was highest in the mixtures of ascorbic acid solution with either raw or boiled vinegar, while that in the mixture containing five per cent acetic acid was lowest. After 24 hours at room temperature the mixtures containing vinegars had lost approximately 85 per cent of the vitamin C present at the time of the immediate test, while the one containing five per cent acetic acid had lost only 15 per cent.

TABLE 1

Effect of Cider Vinegar and Five Per Cent Acetic Acid on Vitamin C Loss in a Solution of Pure Ascorbic Acid (Cebione)

Mixtures	Vitamin C in mixture	
	Immediate test	After 24 hours
	mg. 50 ml.	*mg. 50 ml.*
10 ml. ascorbic acid sol.[1] + 40 ml. distilled water[2]	8.14	5.14
10 ml. ascorbic acid sol.[1] + 40 ml. 5% acetic acid[2]	8.75	7.44
10 ml. ascorbic acid sol.[1] + 40 ml. raw cider vinegar[2]	7.29	1.07
10 ml. ascorbic acid sol.[1] + 40 ml. boiled cider vinegar[2]	6.86	.95

[1] Contents of 0.1-gm. ampul dissolved in 100 ml. of distilled water. [2] Mixtures kept in glass-stoppered volumetric flasks at room temperature.

A later experiment, Clayton (1940), carried out with mixtures of an eight-per cent acetic acid extract of raw green pepper and five per cent acetic acid, or malt, cider, and white vinegars (adjusted to five per cent acidity), again showed a much slower rate of vitamin C loss in the mixture containing the acetic acid than in those containing the vinegars. Also rate of loss varied somewhat with the different vinegars, the mixture containing the white vinegar showing the lowest loss and the mixture containing the cider vinegar the highest loss.

In continuation of these experiments with vinegars recent studies have been made of vitamin C losses in individual cabbage salads made with French dressing containing different kinds of vinegar (three and five per

[1] Merck's Cebione.

cent acidity[2], acetic acid (three and five per cent acidity), or distilled water. Losses in salads made with plain vinegars (three and five per cent acidity[2]), or acetic acid (three and five per cent acidity)[2] also have been studied. Comparisons have been made between the results obtained when the salads were mixed in white enameled bowls with a metal-tined kitchen fork and in glass bowls with a silver fork. The results of these experiments are reported in this paper.

EXPERIMENTAL PROCEDURE

A supply of new early cabbages was bought from a dealer in February, 1945, and kept in cold storage at approximately 1.1°C.(34°F.). Delivery to the laboratory building was made once a week and the cabbages kept in a cool cellar storeroom until used.

Method of Sampling and Analysis for Vitamin C: A different cabbage was used for each day's series of tests. The cabbage was first cut into quarters (down through the core) and a sufficient amount of the tender part of the head was then shredded with a sharp stainless-steel knife. The width of the shreds was about one-eighth inch. The total amount of shredded cabbage was then thoroughly mixed in a large glass bowl and the samples removed from the mixed lot.

For the raw analysis a 50-gram sample was blended for three minutes in a Waring blendor with 200 ml. of three per cent metaphosphoric acid. Two samples of approximately 19 gm. each of the blended mixture were then quickly dipped out of the blendor with an aluminum cream dipper and weighed in previously weighed weighing bottles. The mixture was quantitatively washed out of each bottle with approximately 10 ml. of three per cent metaphosphoric acid into a small mortar (3½ inches in diameter) and ground for two minutes with five gm. of Merck's reagent sea sand. It was then washed from the mortar with about 15 ml. more acid into a 50-ml. centrifuge tube (with constricted neck), stoppered, and centrifuged for 10 minutes. The fairly clear supernatant liquid was poured into a 100-ml. volumetric flask. The residue in the centrifuge tube was extracted twice more with shaking, using approximately 20 ml. of three per cent metaphosphoric acid each time and adding the supernatant liquid to the flask. The extract in the flask was then made up to volume, shaken, filtered through S and S No. 560 fluted paper and titrated with a solution of dichlorobenzenone-indophenol. A five-ml. microburette was used and the indicator was standardized against Merck's Cebione.

The individual salads were made in either white enameled or glass bowls using 50 gm. of cabbage and 10 gm. of French dressing[3] or an equivalent amount of vinegar or acetic acid (3.4 gm.) in each. Mixing was done with either a metal-tined kitchen fork or a silver fork. The bowls were covered with waxed paper and allowed to stand at room temperature [20

[2] Studies were also made of the effect of adding the seasonings used in plain French dressing to plain cider vinegar (five per cent acidity) or to five per cent acetic acid before mixing with the cabbage.

[3] Recipe for French dressing: ½ cup vinegar (121 gm.), 1 cup Wesson oil (217 gm.), 1½ teaspoons salt, 1½ teaspoons sugar, ¾ teaspoon mustard, ½ teaspoon paprika, ¼ teaspoon onion juice, dash of red pepper, and ⅛ teaspoon Worcestershire sauce.

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to 22.5°C.(68 to 72.5°F.)] for the required time intervals. The same method of analysis was used as for the plain raw cabbage, the salads being washed from the bowls into the blendor with 200 ml. of three per cent metaphosphoric acid. The acidity of the vinegars was determined by titration with standard NaOH and necessary dilutions were made with distilled water.

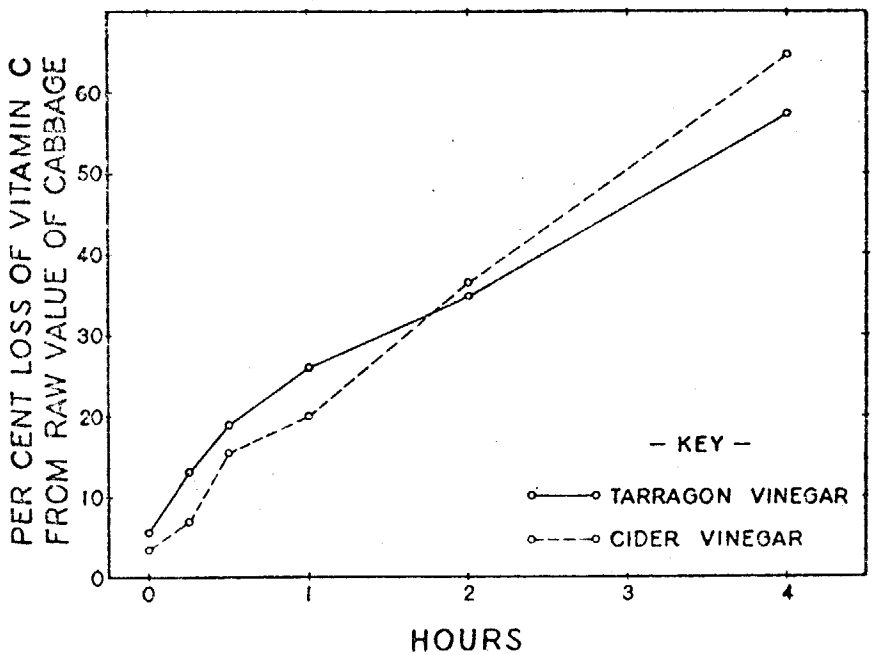

FIG. 1. Rate of loss of vitamin C in the cabbage portion of salads (white enameled bowls and metal-tined kitchen fork used) made with French dressing containing cider or tarragon vinegar of five per cent acidity.

RESULTS

The results of the experiments on cabbage are summarized (Fig. 1 and Tables 2, 3, 4, and 5).

In cabbage salads made with French dressing containing cider or tarragon vinegar of five per cent acidity (Fig. 1) the cabbage lost vitamin C quite rapidly after the addition of dressing containing either kind of vinegar.[4] The immediate loss was somewhat more rapid with the tarragon vinegar dressing but was higher after four hours with the cider vinegar dressing. At the end of two hours the loss was approximately 35 per cent with both kinds of dressing. In this same time interval the loss in the same amount of plain shredded cabbage in the same kind of bowl was only 0.5 per cent.

[4] Similar results were obtained for kale when it was mixed with French dressing containing cider vinegar of five per cent acidity. See Maine Agr. Expt. Sta. Bull. 438, Report of progress for year ending June 30, 1945.

The effect of kind and acidity of vinegar (or acetic acid), when used in French dressings, on the average rate of loss of vitamin C in cabbage salads is shown (Table 2). After two hours the percentage losses of vitamin C were very similar in salads made with French dressing containing the different kinds of vinegar of the same acidity. Also the losses with acetic acid were similar to those with vinegars of the same acidity. In two hours the average percentage loss of vitamin C in salads made with French dressing containing five per cent vinegars or acetic acid was 34.3 per cent. In those containing three per cent vinegar or acetic acid the loss was 21.5 per cent, and in salads made with a control mixture containing distilled water instead of the vinegar of the French dressing the loss was only 0.8 per cent. These differences associated with relative acidity were shown to be highly significant statistically. Enameled bowls and a kitchen fork were used in this series.

TABLE 2

Effect of Kind and Acidity of Vinegar (or Acetic Acid) When Used in French Dressings on Average Rate of Loss of Vitamin C in Cabbage Salads[1]

Kind and acidity of vinegar	Number of tests	Raw cabbage, immediate test	Cabbage in salad, immediate test	Loss from raw value	Cabbage in salad, 2-hr. test	Loss from raw value
		Vitamin C	Vitamin C[2]		Vitamin C[2]	
		mg./100 gm.	*mg./100 gm.*	*pct.*	*mg./100 gm.*	*pct.*
Cider, 5%	2	50.31	48.64	3.4	33.78	32.7
Distilled white, 5%	2	33.39	32.24	3.1	20.97	37.9
Malt, 5%	2	39.89	39.05	2.1	27.68	30.8
Tarragon, 5%	2	33.54	31.66	5.5	20.72	38.3
Acetic acid, 5%	2	46.69	46.03	1.5	32.04	31.4
Average	....	40.76	39.52	3.1	27.04	34.3
Cider, 3%	2	40.73	38.09	6.4	32.53	20.3
Distilled white, 3%	2	34.88	33.72	3.1	27.44	21.9
Malt, 3%	2	31.37	31.09	1.2	24.75	21.4
Tarragon, 3%	2	33.54	31.38	6.4	25.70	23.3
Acetic acid, 3%	2	40.86	40.36	1.5	32.50	20.7
Average	....	36.28	34.93	3.7	28.58	21.5
Distilled water (instead of vinegar in dressing)	2	36.15	34.83	3.7	35.88	0.8

[1] White enameled bowls and metal-tined kitchen fork used in this series. [2] Results calculated on basis of original weight of cabbage in individual salads (50 gm.); 10 gm. dressing in each.

The effects of seven different kinds of plain vinegars and acetic acid (all diluted to three per cent acidity)[5] on average vitamin C losses in cabbage salads are shown (Table 3). In these experiments 3.4 gm. of diluted vinegar or three per cent acetic acid were used with 50 gm. of cabbage. This is the amount of vinegar contained in 10 gm. of French dressing. A statistical study of the results obtained in the two-hour tests indicates that, while there was some variability in the results with the different vinegars, these differences were not significant. Also the results with the three per

[5] All of the vinegars in this series were diluted to three per cent acidity because some were below five per cent as purchased.

cent acetic acid were not significantly different from those with the vinegars. Glass bowls and a silver fork were used in this series.

TABLE 3

Effect of Different Kinds of Plain Vinegars (and Acetic Acid) Diluted to Three Per Cent Acidity, on Average Vitamin C Losses in Cabbage Salads[1]

Kind of vinegar	Number of tests	Raw cabbage, immediate test	Cabbage in salad, immediate test	Loss from raw value	Cabbage in salad, 2 hr. test	Loss from raw value
		Vitamin C	Vitamin C[2]		Vitamin C[2]	
		mg./100 gm.	*mg./100 gm.*	*pct.*	*mg./100 gm.*	*pct.*
Cider	2	23.28	22.50	3.5	15.39	33.9
Distilled white	2	23.28	21.83	6.3	15.13	35.1
Malt	2	23.93	22.82	4.8	17.32	28.0
Tarragon	2	27.92	26.66	4.5	17.60	37.0
Herb salad	2	27.90	25.40	8.5	19.47	30.3
Red wine	2	20.76	19.39	6.6	13.71	34.0
Sauterne	2	20.76	18.69	9.7	12.68	39.0
Acetic acid	2	42.41	39.96	6.2	26.94	37.1
Average	....	26.28	24.66	6.3	17.28	34.3

[1] Glass bowls and silver fork used in this series. Vinegar or acid diluted with glass distilled water. [2] Results calculated on basis of original weight of cabbage in individual salads (50 gm.). Each salad contained 3.4 gm. vinegar (the amount in 10 gm. French dressing).

Effects of utensils used in making cabbage salads containing plain vinegars (or acetic acid) on the average rate of loss of vitamin C are indicated (Table 4). After two hours the percentage losses of vitamin C were 12.7 per cent higher in the salads made with plain five per cent cider vinegar or acetic acid when enameled bowls and a kitchen fork were used instead of glass bowls and a silver fork. When the salads were made with three per cent vinegar the differences owing to the utensils averaged only 4.5 per cent.

Effects on average losses of vitamin C of the addition of seasonings to cider vinegar or acetic acid used in cabbage salads are presented (Tables 2 and 4). A comparison of the results indicates that the cabbage in salads made with plain five per cent cider vinegar lost 34.6 per cent more vitamin C than that in salads made with French dressing containing the same amount of the same kind of vinegar (enameled bowls and kitchen fork used). Similar comparative results were secured with five per cent acetic acid and French dressing containing the same strength of acetic acid. It therefore appears that the oxidizing effect of the vinegar or acid is retarded by other ingredients in the French dressing.

In an attempt to determine whether it was the oil or the seasonings which had the retarding effect on oxidation a series of tests was carried out using plain vinegar (five per cent acidity) or five per cent acetic acid plus the seasonings used in the French dressing. The results of these experiments are given (Table 5). Comparison is made with results previously obtained with plain cider vinegar or acetic acid and with French dressing containing cider vinegar or acetic acid. It is apparent that vitamin C losses were much lower in the salads made with the vinegar or acetic

TABLE 4

Effect of Utensils Used in Making Cabbage Salads Containing Plain Vinegar (or Acetic Acid)[1] *on Average Rate of Loss of Vitamin C*

Acidity of vinegar or acetic acid	Type of bowl and fork	Number of tests	Raw cabbage, immediate test	Cabbage in salad, immediate test	Loss from raw value	Cabbage in salad, 2-hr. test	Loss from raw value
			Vitamin C	Vitamin C[2]		Vitamin C[2]	
			mg./100 gm.	*mg./100 gm.*	*pct.*	*mg./100 gm.*	*pct.*
Cider, 5%	Enameled bowl and kitchen fork	2	36.31	34.28	6.0	11.76	67.3
Acetic acid, 5%	Enameled bowl and kitchen fork	2	39.10	36.04	8.2	12.56	67.4
Average	.....	....	37.71	35.16	7.1	12.16	67.4
Cider, 5%	Glass bowl and silver fork	3	31.42	29.61	5.3	14.86	53.1
Acetic acid, 5%	Glass bowl and silver fork	3	33.15	31.42	5.2	14.10	56.3
Average	.....	....	32.29	30.52	5.3	14.48	54.7
Cider, 3%	Enameled bowl and kitchen fork	2	36.31	32.64	10.3	22.45	38.4
Cider, 3%	Glass bowl and silver fork	2	23.28	22.50	3.5	15.39	33.9

[1] Each salad contained 3.4 gm. of vinegar or acid (the amount in 10 gm. French dressing). [2] Results calculated on basis of original weight of cabbage.

TABLE 5

Effect on Average Losses of Vitamin C of Addition of Seasonings to Cider Vinegar or Acetic Acid Used in Cabbage Salads

Kind of dressing	Number of tests	Raw cabbage, immediate test	Cabbage in salad, immediate test	Loss from raw value	Cabbage in salad, 2-hr. test	Loss from raw value
		Vitamin C	Vitamin C[1]		Vitamin C[1]	
		mg./100 gm.	*mg./100 gm.*	*pct.*	*mg./100 gm.*	*pct.*
Cider vinegar, 5%[2]	3	31.42	29.61	5.3	14.86	53.1
Acetic acid, 5%[2]	3	33.15	31.42	5.2	14.10	56.3
Cider vinegar, 5%, plus seasonings[2]	2	46.13	44.46	4.0	34.16	27.1
Acetic acid, 5%, plus seasonings[2]	2	38.43	37.58	2.2	28.83	25.1
French dressing[2] with 5% cider vinegar	2	50.31	48.80	3.0	31.31	25.8
French dressing[2] with 5% acetic acid	2	46.69	46.22	1.0	34.46	26.2

[1] Results calculated on basis of original weight of cabbage in individual salads. Each salad contained 10 gm. French dressing or the equivalent amount of vinegar or vinegar plus seasonings. [2] Glass bowls and silver fork used. [3] Since enameled bowls and kitchen fork were used for the salads containing French dressing, the values given have been corrected to make them comparable to those given for the vinegar and acetic acid and the vinegar and acetic acid plus seasonings.

acid containing the seasonings than in those containing vinegar or acid alone. The losses in salads made with French dressing were almost identical with those in salads made with equivalent amounts of the vinegar or acid plus seasonings. (Analysis of the freshly mixed seasonings showed that they contained only a trace of vitamin C.) Therefore it appears that the lower losses of vitamin C which occur in cabbage salads made with French dressing (as compared with those made with plain vinegar) are chiefly due to the seasonings in the dressing rather than the oil.

DISCUSSION

According to Wachholder (1942) the vitamin C in cabbage can be oxidized by a heat-labile ascorbic acid oxidase and by heat-stable metal containing catalysts. Bach (1945) states that at a pH of 6.5 an aqueous vapor distillate of cabbage is inhibitory to the catalytic effect (on vitamin C oxidation) of $CuSO_4 \cdot 5H_2O$ in a concentration of 5.7×10^{-5} M but not at a concentration of 5.7×10^{-4} M. She explains this inhibitory action by the fact that the distillate contains sulphur compounds which probably combine with copper to form sulfides.

In the experiments reported in the present paper the damage to the cells of the cabbage during shredding with a stainless-steel knife was apparently not great enough to release the ascorbic acid oxidase. Therefore the protective substances present in the cabbage were adequate to protect the vitamin C in the plain shredded cabbage and in the cabbage mixed with the French dressing containing distilled water instead of vinegar (Table 2). However, when vinegar or acetic acid was added to the cabbage, either plain or in French dressing, metallic ions were probably liberated which catalyzed the oxidation. The source of part of these ions was probably in the traces of metallic salts which were present as contamination. Also the increase in acidity was apparently sufficient to free other metallic ions which had previously been combined with the protective substances. This explanation appears logical, since the loss of vitamin C in the cabbage was proportional to the titratable acidity of the vinegar or acetic acid.

The theoretical pH of five per cent acetic acid is 2.4 and of three per cent, is 2.5. According to Tauber, Kleiner, and Mishkind (1935) inactivation occurs in purified squash oxidase at pH 2. Therefore it does not seem probable that the ascorbic acid oxidase in the cabbage was activated by the vinegar or acetic acid used in the cabbage salads.

The increased oxidation of vitamin C which occurred when the enameled bowls and kitchen fork were used in the presence of vinegar or acetic acid can be explained as being due to the effect of additional metallic ions from the bowls and fork.

Strohecker, Busse, and Weinreich (1941) found that of a number of metals which they tested silver and copper had the strongest action in catalyzing vitamin C oxidation. Therefore, it seemed possible that the silver fork, used in some of the experiments reported in the present paper, might have provided silver ions which were partly responsible for the vitamin C oxidation. However, in a series of experiments in which a comparison was made of the effect of mixing cabbage salads containing French

dressing with a recently polished silver fork or with one of a transparent plastic material, no significant difference was shown. In these experiments the salads were mixed in glass bowls and held at room temperature for one- and two-hour intervals before testing.

The question arises as to why there was a significant difference between the results with vinegars and those with acetic acid in the experiments with pure ascorbic acid and in those with extract of green pepper but none in the cabbage experiments. The answer to this question may be given by pointing out certain differences in the conditions existing in the different experiments. In the experiment with the solution of pure ascorbic acid (Table 1) the only metallic ions (such as iron or copper) introduced were those in the vinegar itself; therefore the oxidation was more rapid in the mixtures containing vinegar. In the experiment with the extract of green pepper in eight per cent acetic acid, Clayton (1940), only a small amount of the pepper extract (probably containing only traces of metallic ions) was used, so the catalytic effect of the metallic ions in the different vinegars (but not in the acetic acid) caused differences in the rate of vitamin C oxidation in the pepper extract.

In the cabbage experiments the amount of cabbage used in proportion to acetic acid or vinegar was large. Since the cabbage itself probably contained traces of metals which could be ionized by acid and since there was also some contamination by traces of metals in the utensils used, a difference between the effect of acetic acid and vinegar was not apparent. It is also possible that if shorter time intervals had been used in the cabbage experiments, differences could have been shown between the effects of the different kinds of vinegar. However, the acidity of the vinegar or acetic acid appears to be the most important factor responsible for loss of vitamin C in the cabbage salads.

The protective effect of the seasonings cannot be definitely explained. They may have contained reducing substances which did not affect the indicator in the vitamin C titrations. Also, they probably decreased the acidity of the vinegar and acetic acid to some extent.

As a practical application of the above findings it is suggested that in order to obtain the maximum amount of vitamin C from cabbage salads containing vinegar or French dressing the vinegar or dressing should be added to the cabbage just before eating. The same suggestion applies to salads made with other greens. Contact of the salads with metals, especially iron and copper, should be avoided as much as possible.

SUMMARY

Vitamin C losses were studied in individual cabbage salads made with different kinds of vinegar (or acetic acid) and with French dressings containing vinegars (or acetic acid). The losses in salads made with French dressing were lower than in those containing the same amount of plain vinegar. After the salads had stood two hours at room temperature the loss of the vitamin was proportional to the titratable acidity of the vinegar (or acetic acid). This was apparently due to the liberation of metallic ions by the acid.

REFERENCES

BACH, ERNA, 1945. Inhibition of the oxidation of ascorbic acid. III. Z. Vitaminforsch. 15, 297-308.

CLAYTON, MARY M., 1940. The effect of sodium chloride and vinegars on the oxidation of ascorbic acid (vitamin C). Rept. of progress for year ending June 30, 1940. Maine Agr. Expt. Sta. Bull. 400, 231-235.

STROHECKER, R., BUSSE, O., AND WEINREICH, 1941. Ueber die Haltbarkeit von l-Ascorbinsäure (Vitamin C). Z. Untersuch. Lebensm. 81, 126.

TAUBER, H., KLEINER, I. S., AND MISHKIND, D., 1935. Ascorbic acid (vitamin C) oxidase. J. Biol. Chem. 110, 211-218.

WACHHOLDER, K., 1942. Vitamin-C-Oxydatoren in pflanzlichen Nahrungsmitteln und Vitamin-C-Verluste bei der küchenmässigen Zubereitung. Ernährung 7, 129-136.

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Roles of Acetate and Pyruvate in the Metabolism of *Streptococcus diacetilactis*

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Streptococcus diacetilactis required acetate, contained acetate kinase and phosphotransacetylase, and incorporated both radioactive exogenous acetate and acetate from citrate into cell lipids. DL-α-Lipoic acid replaced acetate and was required for the oxidation of pyruvate. Stimulation of *S. diacetilactis* by citrate was found to depend on pyruvate oxidation. Resting cells of the organism produced acetate from 73% of the pyruvate they utilized. However, molar growth yields from glucose were not greater under aerobic compared to anaerobic conditions or when lipoic acid or citrate plus lipoic acid was used in the medium in place of acetate. Data indicate that the growth of *S. diacetilactis* is limited by the rate of acetyl-coenzyme A synthesis, that the rate of synthesis from pyruvate is higher than the rate from acetate, and that lack of acetyl-coenzyme A not required for growth limits the production of diacetyl and precludes the formation of adenosine triphosphate from acetyl-coenzyme A.

Collins et al. (5) found that the partially defined medium devised by Niven (27) required addition of acetate or Reticulogen, a refined liver preparation, to support the growth of several strains of *Streptococcus lactis* and *S. cremoris*, organisms closely related to *S. diacetilactis*. Reed et al. (31) found that the growth factor in liver that substituted for acetate in media for *S. lactis* was α-lipoic acid.

Either acetate or pyruvate can serve as a precursor of acetyl-coenzyme A (CoA). From pyruvate, following decarboxylation and formation of an acetaldehyde-thiamine pyrophosphate (TPP) complex, lipoic acid is involved in transfer of the acyl group of the acetaldehyde-TPP complex to CoA (6, 10, 11, 28, 29). From acetate, there are two mechanisms for the formation of acetyl-CoA. One mechanism occurs in animal tissues, yeast, and some bacteria and involves a single enzyme, acetyl-CoA synthetase (17). The other, restricted to bacteria, involves two enzymes, acetate kinase (19) and phosphotransacetylase (39).

This work was undertaken to define the acetate requirement of *S. diacetilactis* and determine the mechanism of acetate utilization by this organism in relation to pyruvate oxidation and the potential for formation of diacetyl and acetoin by known mechanisms (4, 38).

MATERIALS AND METHODS

Organism. The organism studied was *S. diacetilactis* DRCI (24, 41), which was propagated routinely at 22 C in litmus milk. Identity of the culture was ascertained periodically by checking it for sensitivity to its homologous bacteriophage. *S. faecalis* strain 10 CI was propagated at 37 C either in litmus milk or on the nutrient agar medium described by Stokstad et al. (40).

Media. The partially defined, lipoic acid-free medium used to grow *S. diacetilactis* was the same as that reported by Harvey and Collins (15), except that 0.4% glucose replaced lactose, citrate was omitted, the casein hydrolysate content was increased to 0.2%, pantothenate (1.0 μg/ml) and pyridoxal (1.2 μg/ml) were included, and the medium was adjusted to *p*H 6.8. [It has been reported that some casein hydrolysates have low levels of methionine (1), but addition of DL-methionine (1.0 μg/ml) to the basal medium did not enhance the growth of *S. diacetilactis*.] In some experiments, DL-α-lipoic acid (1.0 μg/ml) replaced sodium acetate (0.2%). The lipoic acid-free basal medium used to grow *S. faecalis* was described by Gunsalus et al. (13). Tryptone broth contained 1.0% tryptone, 1.0% yeast extract, 0.5% K_2HPO_4, and 0.1% glucose, and was adjusted to *p*H 6.8.

Inocula. Cell suspensions prepared as follows were used as inocula in experiments to determine the acetate-replacing ability of various chemicals and the growth responses of *S. diacetilactis* to acetate or lipoic acid. Cells were harvested from the basal medium (tryptone broth in one experiment) by centrifugation, washed three times in 0.05 M potassium phosphate buffer (*p*H 6.8), and suspended in basal medium without acetate. Enough cell suspension was added to each flask or test tube of an experiment to give an initial optical density (OD_{600}) at 600 nm of 0.01 or 0.02. Growth was considered to be negative in those

flasks or tubes in which the turbidity did not increase during incubation for 36 hr at 32 C.

Measurement of growth. Bacterial growth was followed by periodically measuring OD at 600 nm with a Beckman spectrophotometer, model DB, with 1-cm cuvettes. Cell mass was determined from a standard curve relating OD to dry weight. An OD value of 3.72 represented 1 mg (dry weight) per ml for *S. diacetilactis* DRCl cells harvested in either the exponential or the stationary phase of growth. This relationship was linear up to at least an OD value of 0.6.

Cultures generally were grown statically at 32 C in an atmosphere of air. Aerobic conditions, where indicated, were accomplished by sparging the medium with sterile air passed through a sterile water train at a rate of 20 ml/min. Anaerobic conditions were obtained by purging with sterile nitrogen prior to inoculation and subsequently bubbling nitrogen (99.999% pure) through the medium at a rate of 1 to 2 ml/min.

Cell-free extracts. Cells of *S. diacetilactis* were harvested from the indicated medium near the end of exponential growth by centrifugation, washed three times with cold 0.01 M tris(hydroxymethyl)aminomethane-cysteine (Tris)-cysteine buffer at *p*H 7.0 (0.001 M with respect to L-cysteine), and resuspended in Tris-cysteine buffer at a cell density of ca. 50 mg (wet weight) per ml. The cells were disrupted with 0.2-mm glass beads (50%, w/w) in a Mickle tissue disintegrator operated at 6- to 8-mm amplitude for 50 min at 4 C. The crude extract was centrifuged at 15,000 × *g* for 40 min at 0 C in a refrigerated centrifuge. The supernatant fluid was decanted, dialyzed against 100 volumes of 0.01 M Tris-cysteine buffer (*p*H 7.0) for 24 hr at 4 C, and stored at 0 or −20 C. For certain indicated experiments, the extracts were treated with anion-exchange resin (acid-washed Dowex 1-X2) to remove CoA (3). [This procedure also removes oxaloacetate decarboxylase (15).]

Analytical procedures. Acetate kinase and phosphotransacetylase activities in cell-free extracts were determined with the hydroxamic acid method of Seamen (35). One micromole of hydroxamic acid corresponded to an absorbancy of 0.14 at 540 nm. A unit of acetate kinase activity is the amount required to produce 1 μmole of hydroxamic acid per min, and specific activity is the number of units per milligram of protein. The reaction exhibited zero-order kinetics for 10 min, and the reaction rate was proportional to concentration of the enzyme up to 0.4 unit per ml. A unit of phosphotransacetylase activity is the amount required to catalyze the arsenolysis of 1 μmole of acetylphosphate per min. Specific activity is number of units per milligram of protein.

Pyruvate oxidation was determined by the method described by Gunsalus et al. (13), which involves measurement of the oxygen consumed by conventional Warburg manometric techniques (43). Oxygen uptake was measured at 37 C for *S. faecalis* and at 32 C for *S. diacetilactis*. The assay systems contained 5 to 6 mg of cells (dry weight). Pyruvic acid was determined by the method of Friedmann and Haugen (9). Acetoin and diacetyl were determined as the sum of both compounds by the method of Westerfeld (45). Citrate was determined by the method of Marier and Boulet (23). Glucose was determined by the modified anthrone method of Ecker and Lockhart (8). The protein in cell-free extracts was determined by the method of Lowry et al. (20) with bovine serum albumin used as the standard.

Radioactivity measurements were made with an SC-16 windowless flow counter and a Versamatic II scaler (Tracerlab Inc., Waltham, Mass.).

Chemicals. Sodium acetate, sodium citrate, sodium arsenite, and potassium arsenate were commercial crystalline reagent grade. The nucleotides, nucleosides, crystalline DL-α-lipoic acid, sodium acetate-*2-*^{14}*C*, sodium citrate-*1,5-*^{14}*C*, the potassium salt of adenosine triphosphate (ATP), adenosine monophosphate (AMP), adenosine, sodium and potassium pyruvate, and the *N,N'*-dibenzylethylene diamine salt of mevalonic acid were obtained from Calbiochem (Los Angeles, Calif.). CoA and lithium acetyl phosphate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Free mevalonic acid was prepared from the salt by adjusting an aqueous solution of the salt to *p*H 11.3 with 5 N NaOH, extracting three times with an equal volume of diethyl ether, and removing the ether in vacuo. Dihydrolipoic acid was prepared from DL-α lipoic acid with sodium borohydride by the method of Gunsalus et al. (12) as modified by Reiss (32). Vitamin-free casein hydrolysate, crystalline albumin bovine plasma, and *N*-ethylmaleimide were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio).

RESULTS

Response to acetate. *S. diacetilactis* required acetate, though occasionally growth occurred in the partially defined medium lacking acetate. Maximal cell yields (0.298 to 0.306 mg of dry cells per ml) were obtained with acetate concentrations of 0.8 to 2.0 mg/ml, maximal specific growth rates (k = 0.658/hr) occurred with concentrations of 1.0 to 4.0 mg/ml, and the shortest lag time (2 hr) occurred with 2.0 mg/ml. A plot of the maximal yield data indicated that the response of *S. diacetilactis* to acetate was linear, similar to that reported for *Lactobacillus acidophilus* (37). Concentrations of acetate above 8.0 mg/ml generally were inhibitory.

Growth in the absence of acetate occurred more often in tests in which the inoculum was large (0.05 mg of dry cells per ml), but those cultures that grew could not be serially transferred in the medium lacking acetate. Cell yields were low, lag periods were long, and specific growth rates were low (k = 0.3/hr). We were unable to isolate a nonacetate-requiring mutant, and sterilization of the medium by filtration did not prevent the occasional growth in the absence of acetate.

Substitutes for acetate. DL-α-lipoic acid, reduced lipoic acid, and natural substances containing lipoic acid (yeast extract and liver ex-

tract) replaced acetate. Maximal cell yields were obtained with DL-α-lipoic acid concentrations of 0.02 ng/ml or greater, and half maximal yields were obtained with 0.01 ng/ml. These concentrations are double those that should have been obtained with L-lipoic acid, since only the L form of lipoic acid is biologically active (16, 44). *S. lactis* and *S. cremoris* required 0.02 ng/ml and 3 to 20 ng/ml, respectively, for half maximal yields (21, 31).

None of the following substances or alterations of the partially defined medium replaced acetate: 10 mg of Tween 80 per ml, 4 mg of sodium citrate per ml, 0.5 μg of mevalonic acid per ml (40), 2 mg of β-alanine per ml, 1.2 mg of sodium pyruvate per ml, 0.1 mg of β-lipoic acid per ml (30), omission of ferrous ions (22), increased glucose (42), increased nucleosides and nucleotides (22), or incubation under anaerobic conditions (36).

Pyruvate oxidation. We tested *S. diacetilactis* to determine whether this organism has a lipoic acid-dependent pyruvate dehydrogenase system similar to that reported for *S. faecalis* (13, 28, 29). *S. diacetilactis* contains reduced nicotinamide adenine dinucleotide (NADH) oxidase (38), so we used the method of Gunsalus et al. (13). Resting cells of *S. diacetilactis* harvested from the basal medium oxidized pyruvate only when lipoic acid was present (Table 1). Arsenite inhibited the oxidation, as it did the oxidation of pyruvate by *S. faecalis* (11). Cells of *S. diacetilactis* or *S. faecalis* harvested from tryptone broth, however, oxidized pyruvate without added lipoic acid (Table 1). That these cells had accumulated lipoic acid during growth in the tryptone broth was indicated by the fact that heated extracts prepared from cells of *S. diacetilactis* harvested from tryptone broth replaced lipoic acid in the oxidation of pyruvate by resting cells of *S. faecalis* (Table 1).

Stoichiometry of the reaction mediated by *S. diacetilactis* was indicated by measurements of the pyruvate and oxygen utilized by cells of the organism harvested from the basal medium. Without addition of lipoic acid to the reaction flasks, the resting cells produced acetoin plus diacetyl from 89% of the pyruvate that was utilized. With 3 μg of lipoic acid added, they utilized 33.3 μmoles of pyruvate, consumed 12.1 μmoles of oxygen, and produced 3.0 μmoles of acetoin plus diacetyl (from 6.0 μmoles of pyruvate). The amount of acetate produced, calculated from the amount of oxygen utilized, was 24.2 μmoles, leaving 3.1 μmoles of pyruvate unaccounted for in the results.

Stimulation of growth. Citrate stimulates the growth rate of *S. diacetilactis* about 35% under some cultural conditions (15). Our results show that stimulation depends on the oxidation of pyruvate (Table 2). *S. diacetilactis* was not stimulated by the addition of citrate (0.1%) to the partially defined lipoic acid-free casein-hydrolysate medium, though an increase in the production of acetoin plus diacetyl showed that the citrate was broken down. With lipoic acid

TABLE 1. *Oxidation of pyruvate by resting cells of S. diacetilactis or S. faecalis*

Source of lipoic acid	Amt (μliters) of oxygen consumed per hr per mg of dry cells		
	S. diacetilactis[a]		*S. faecalis*
	A	B	
None	0	0	0
DL-α-Lipoic acid (10 μg)	16	0	135
Residual from tryptone broth[b]	14	0.5	64[c]
Heated extract of *S. diacetilactis* cells harvested from basal medium (1.4 mg of protein)			0
Heated extract of *S. diacetilactis* cells harvested from tryptone broth (2.0 mg of protein)			81

[a] A, no addition; B, sodium arsenite (20 μmoles) added.

[b] The cells used in these assays were harvested from tryptone broth.

[c] With sodium arsenite (20 μmoles) added, 1.7 μliters of oxygen were consumed per hr per mg of dry cells.

TABLE 2. *Influence of lipoic acid on the growth rate of S. diacetilactis*[a]

Medium alteration[b]	Specific growth rate per hr	Citrate used (μmoles/mg, dry wt)	Acetoin plus diacetyl produced (μmoles/mg, dry wt)
None	0.682		7.8
Plus citrate (0.1%)	0.546	9.7	20.1
Plus sodium lipoate (10 μg/ml); minus sodium acetate	0.768		0.0
Plus sodium lipoate (10 μg/ml) and sodium citrate (0.1%); minus sodium acetate	0.785		

[a] Growth temperature was 32 C.

[b] Unaltered medium was the partially defined casein-hydrolysate medium, pH 6.5.

added to the medium in place of acetate, pyruvate produced from glucose could be oxidized, and the organism was stimulated whether or not citrate was added.

Cell yields. Resting cells of *S. diacetilactis* converted pyruvate to acetate as did cells of *S. faecalis*, but previous experiments had indicated that *S. diacetilactis* differs from *S. faecalis* by not gaining energy from utilization of citrate or pyruvate (7, 15, 25, 26). Present results substantiated earlier findings. With glucose limited in the basal medium, cell yields per mole were not significantly greater under aerobic compared to anaerobic conditions, with lipoic acid used in place of acetate, or with citrate plus lipoic acid used in place of acetate.

Activation of acetate. Dialyzed cell-free extracts of *S. diacetilactis* that had been treated with Dowex 1 to remove CoA activated acetate, and the activation was dependent on ATP. Addition of 12.5 units of CoA to the reaction mixture did not stimulate the activation, indicating that this organism contains acetate kinase rather than acetyl-CoA synthetase. Utilization of acetyl phosphate by the extracts depended upon the addition of CoA, showing that they contained phosphotransacetylase. The acetate kinase and phosphotransacetylase activities of extracts prepared from cells grown in tryptone broth were as great as those of extracts prepared from cells grown in the presence of acetate, indicating that these enzymes of *S. diacetilactis* are constitutive.

Properties of acetate kinase. The properties of the acetate kinase of *S. diacetilactis* were similar in most respects to those reported for the acetate kinases of other organisms. The optimal *p*H for its activity in dialyzed Dowex 1 treated cell-free extracts of cultures grown in the partially defined medium, tryptone broth, and tryptone broth plus 0.2% acetate was 7.4, similar to that reported for *Escherichia coli* and *S. hemolyticus* (30) and *Desulfovibrio desulfuricans* (2), somewhat above that reported for *Clostridium acidi-urici* (34). Treatment of the extracts with ethylenediaminetetraacetate or omission of divalent metal ions from the reaction mixture prevented the activation of acetate. Mg^{2+} and Mn^{2+} were equally effective in the assay system, Co^{2+} was only 35% as effective as Mg^{2+} or Mn^{2+}, and Ca^{2+} was ineffective.

None of the following replaced acetate in the reaction: citrate (640 μmoles), butyrate (160 μmoles), propionate (640 μmoles), or formate (160 μmoles), similar to the findings for *D. desulfuricans* (2). Neither adenosine diphosphate nor AMP replaced ATP in the forward reaction, substantiating the findings of Rose et al. (33). *N*-ethylmaleimide (5 μmoles) inhibited the enzyme, and addition of L-cysteine (10 μmoles) prior to the addition of *N*-ethylmaleimide gave protection, indicating, as did the results of Rose et al. (33) and Brown and Akagi (2), that acetate kinase has an essential SH group.

The half-saturation concentrations (K_m), determined by conventional Lineweaver-Burk plots (18) in 1.0 M Tris buffer with extracts of cells grown in the basal medium, tryptone broth, and tryptone broth plus 0.2% acetate, were 0.136 M, 0.145 M, and 0.136 M, respectively. These values were close to the value reported for *E. coli* (33) but different from that found for *D. desulfuricans* (4.5×10^{-4} M; reference 2). Specific activities of the acetate kinase in extracts prepared from cells grown aerobically and anaerobically in a variety of media were essentially the same, indicating that neither the presence of oxygen during growth nor complexity of the growth medium greatly influenced the level of acetate kinase activity.

Incorporation of acetate into cell material. *S. diacetilactis* was grown in the following medium supplemented with 1.0 μc of acetate-2-^{14}C (37,000 counts/sec) with and without the addition of 0.5% citrate: 0.25% lactose, 0.25% casein hydrolysate, 0.25% yeast extract, 0.50% KH_2PO_4, 0.10% $NaHCO_3$, 0.02% $MgSO_4$, and 0.05% acetate, adjusted to *p*H 6.4. Near the end of exponential growth, the cells from each culture were harvested by centrifugation and washed once with a citrate-lactate-acetoin solution (0.25% of each), once with a pyruvate-bicarbonate solution (0.25% of each), and twice with distilled water. The cells were then suspended in water, and samples were plated for counting. The remaining cells were dried, and the lipids were extracted with hot 95% ethanol by the procedures described by Harvey and Collins (15).

Radioactivity measurements showed that the cells had incorporated acetate into their lipids. Cells that had been grown in the absence of citrate incorporated an amount of radioactive carbon equal to 1.78 μmoles of acetate per mg of cells (dry weight). Those that had been grown in the presence of citrate incorporated a smaller amount of radioactive carbon, as was expected since acetate and pyruvate are products of citrate (14) and should have diluted the radioactivity. The amount incorporated was equal to 0.5[illegible] μmole of acetate per mg of cells (dry weight). That acetate from the citrate was in fact incorporated into cell material was established in a similar experiment in which the medium containing 0.5% citrate was supplemented with 1.0 μc of citrate-*1,5*-^{14}C. Radioactivity from the citrate equal to 2.18 μmoles of acetate per mg of cells (dry weight) was found in the lipid fraction.

DISCUSSION

The first steps in the synthesis of fatty acids from pyruvate or acetate involve the formation of acetyl-CoA. Results indicate that *S. diacetilactis* forms acetyl-CoA from pyruvate by means of a lipoic acid-dependent dehydrogenase system [similar to that reported for *S. faecalis* (13, 28, 29)] or from acetate by means of acetate kinase and phosphotransacetylase. With lipoic acid in the partially defined casein hydrolysate medium in place of acetate, the organsim obviously secured sufficient pyruvate for the synthesis of acetyl-CoA, probably from the metabolism of glucose. That pyruvate not required for oxidation of NADH or cell synthesis was derived from the medium containing acetate was indicated by the formation of 7.8 μmoles of acetoin plus diacetyl (Table 2).

Resting cells of *S. diacetilactis* in the presence of lipoic acid formed acetoin plus diacetyl from only 18% of the pyruvate that was utilized. Most of the remainder (73% of the total), determined from the amount of oxygen utilized in oxidation of the NADH that was formed in the reoxidation of lipoic acid, was converted via acetyl-CoA to acetate. Not more than 9% of the pyruvate that was utilized could have been converted to acetaldehyde since the formation of acetaldehyde from acetyl-CoA would have resulted in a proportional decrease in the consumption of oxygen. This oxidation of pyruvate to acetate probably involved the actions of phosphotransacetylase and the ATP-dependent acetate kinase that we have found the organism to possess. Yet, we found that the cell yield of *S. diacetilactis*, determined under aerobic conditions in the basal medium containing a growth-limiting amount of glucose, was not increased by the addition of lipoic acid plus citrate as a source of pyruvate. These results, which are supported by the results of Harvey and Collins (15), indicate that the growing cells did not get from acetyl-CoA enough ATP to influence molar growth yields. Since the organism has an ATP dependent acetate kinase, we interpret the results to indicate that in growing cells of *S. diacetilactis* the amount of acetyl-CoA that is available for use in the formation of acetate is severely limited.

The growth rate of *S. diacetilactis* was increased by the substitution of lipoic acid for acetate in the partially defined medium, suggesting that the rate of acetyl-CoA synthesis from pyruvate is higher than the rate from acetate. Nevertheless, the organism incorporated into cell lipids both acetate from citrate-*1,5-*^{14}*C* and, also, exogenous acetate-*2-*^{14}*C* from a medium that contained citrate. In each of these cases the medium contained 0.25% yeast extract as a source of lipoic acid. This use of acetate as a precursor of acetyl-CoA in the presence of pyruvate and lipoic acid indicates that pyruvate oxidation did not meet entirely the biosynthetic demands of *S. diacetilactis* for acetyl-CoA.

Our results and published molar growth yield data (15, 25) indicate that in growing cells of *S. diacetilactis* very little, if any, of the acetyl-CoA formed from pyruvate is available for the production of ATP. Other data (15) show that excess pyruvate that is not needed for oxidation of NADH or cell synthesis is converted primarily to acetoin plus diacetyl. Together, these results suggest that the formation of acetyl-phosphate from acetyl-CoA is limited in growing cells of this organism and, consequently, that any acetyl-CoA not required for cell synthesis is used primarily for the production of diacetyl rather than acetate. This possibility, however, is not supported by the results showing that resting cells produced acetate from 73% of the pyruvate they utilized.

The production of acetoin does not involve acetyl-CoA, but the production of diacetyl depends on the availability of acetyl-CoA not required for cell synthesis (4, 38). Numerous reports show that *S. diacetilactis* and other organisms that produce diacetyl produce much smaller amounts of it than they do of acetoin, and some microorganisms that produce acetoin do not produce detectable diacetyl (4, 38). Such findings suggest that growing cells of several species besides *S. diacetilactis* produce little acetyl-CoA in excess of that required for cell synthesis.

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LITERATURE CITED

1. Brodovsky, E. R., M. H. Urley, and W. H. Pearson. 1958. Methionine inadequacy of casein hydrolysate as a difficulty in vitamin assay. Science 128:307-308.
2. Brown, M. S., and J. M. Akagi. 1966. Purification of acetokinase from *Desulfovibrio desulfuricans*. J. Bacteriol. 92:1273-1274.
3. Chantrenne, H., and F. Lipmann. 1950. Coenzyme A dependence and acetyl donor function of the pyruvate-formate exchange system. J. Biol. Chem. 187:757-767.
4. Chuang, L. F., and E. B. Collins. 1968. Biosynthesis of diacetyl in bacteria and yeast. J. Bacteriol. 95:2083-2089.
5. Collins, E. B., F. E. Nelson, and C. F. Parmele. 1959. Acetate and citrate requirement of the lactic group of streptococci. J. Bacteriol. 77:69-74.
6. Das, M. L., M. Koike, and L. J. Reed. 1961. On the role of thiamin pyrophosphate in oxidative decarboxylations of α-keto acids. Proc. Nat. Acad. Sci. U.S.A. 47:753-759.
7. Deibel, R. H., and C. F. Niven, Jr. 1964. Pyruvate fermentation by *Streptococcus faecalis*. J. Bacteriol. 88:4-10.
8. Eckert, R. E., and W. R. Lockhart. 1961. Specific effect of limiting nutrient on physiological events during culture growth. J. Bacteriol. 82:511-516.

9. Friedmann, T. E., and G. E. Haugen. 1943. Pyruvic acid; determination of keto acids in blood and urine. J. Biol. Chem. 147:415-442.

10. Gunsalus, I. C. 1954. Group transfer and acyl-generating functions of lipoic acid derivatives, p. 545-580. *In* W. D. McElroy and B. Glass (ed.), Mechanism of enzyme action. John Hopkins Univ. Press, Baltimore.

11. Gunsalus, I. C. 1954. Oxidative and transfer reactions of lipoic acid. Fed. Proc. 13:715-722.

12. Gunsalus, I. C., L. S. Barton, and W. Gruber. 1956. Biosynthesis and structure of lipoic acid derivatives. J. Amer. Chem. Soc. 78:1763-1766.

13. Gunsalus, I. C., M. I. Dolin, and L. Struglia. 1952. Pyruvic acid metabolism. III. A manometric assay for pyruvate oxidation factor. J. Biol. Chem. 194:849-857.

14. Harvey, R. J., and E. B. Collins. 1961. Role of citritase in acetoin formation by *Streptococcus diacetilactis* and *Leuconostoc citrovorum*. J. Bacteriol. 82:954-959.

15. Harvey, R. J., and E. B. Collins. 1963. Roles of citrate and acetoin in the metabolism of *Streptococcus diacetilactis*. J. Bacteriol. 86:1301-1307.

16. Hornberger, C. S., Jr., R. F. Heitmiller, I. C. Gunsalus, G. H. F. Schnakenberg, and L. J. Reed. 1953. Synthesis of D,L-alpha lipoic acid. J. Amer. Chem. Soc. 75:1273-1277.

17. Jones, M. E., S. Black, R. M. Flynn, and F. Lipmann. 1953. Acetyl coenzyme A synthesis through pyrophosphoryl split of adenosine triphosphate. Biochim. Biophys. Acta 12:141-149.

18. Lineweaver, H., and D. Burk. 1934. The determination of enzymatic dissociation constants. J. Amer. Chem. Soc. 56:658-666.

19. Lipmann, F. 1944. Enzymatic synthesis of acetyl phosphate. J. Biol. Chem. 155:55-70.

20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

21. Lytle, V. L., and D. J. O'Kane. 1951. The pyruvate oxidation factor requirement of Streptococcus cremoris. J. Bacteriol. 61:240-241.

22. MaciasR, F. M. 1959. Acetate stimulation of *Lactobacillus delbrueckii* and its replacement by nucleosides. J. Bacteriol. 77:497-505.

23. MaciasR, J. R., and M. Boulet. 1958. Direct determination of citric acid in milk with an improved pyridine-acetic anhydride method. J. Dairy Sci. 41:1683-1692.

24. Matuszewski, T., E. Pijanowski, and J. Supinski. 1936. *Streptococcus diacetilactis* n. sp. i jego zastosowanie przy wyrobie masla. Rocz. Nauk Roln. Ser. B 36:1.

25. Moustafa, H. H., and E. B. Collins. 1968. Molar growth yields of certain lactic acid bacteria as influenced by autolysis. J. Bacteriol. 96:117-125.

26. Moustafa, H. H., and E. B. Collins. 1969. Molar growth yield of *Streptococcus faecalis* on pyruvate. J. Bacteriol. 97:1496-1497.

27. Niven, C. F., Jr. 1944. Nutrition of Streptococcus lactis. J. Bacteriol. 47:343-350.

28. O'Kane, D. J. 1950. Influence of the pyruvate oxidation factor on the oxidative metabolism of glucose by Streptococcus faecalis. J. Bacteriol. 60:449-458.

29. O'Kane, D. J., and I. C. Gunsalus. 1948. Pyruvic acid metabolism. A factor required for oxidation by Streptococcus faecalis. J. Bacteriol. 56:499-506.

30. Reed, L. J., B. G. DeBusk, C. S. Hornberger, and I. C. Gunsalus. 1953. Interrelationships of lipoic acids. J. Amer. Chem. Soc. 75:1271-1273.

31. Reed, L. J., B. G. DeBusk, P. M. Johnson, and M. E. Gelzer-daner. 1951. Acetate-replacing factors for lactic acid bacteria. I. Nature, extraction, and distribution. J. Biol. Chem. 192:851-858.

32. Reiss, O. K. 1958. Pyruvate utilization in heart sacrasomes by (+)-lipoic acid. J. Biol. Chem. 233:789-793.

33. Rose, I. A., M. Grunberg-Manago, S. R. Korey, and S. Ochoa. 1954. Enzymatic phosphorylation of acetate. J. Biol. Chem. 211:737-756.

34. Sagers, R. D., M. Benziman, and I. C. Gunsalus. 1961. Acetate formation in *Clostridium acidi-urici*: acetokinase. J. Bacteriol. 82:235-238.

35. Seaman, G. R. 1963. Experiments in microbial physiology and biochemistry. Burgess Publishing Co., Minneapolis.

36. Shockman, D. G. 1956. The acetate requirement of *Streptococcus faecalis*. J. Bacteriol. 72:101-104.

37. Snell, E. E., M. Guirard, and R. J. Williams. 1946. The nutritional role of acetate for lactic acid bacteria. I. Its response to substances related to acetate. Arch. Biochem. 9:361-379.

38. Speckman, R. A., and E. B. Collins. 1968. Diacetyl biosynthesis in *Streptococcus diacetilactis* and *Leuconostoc citrovorum*. J. Bacteriol. 95:174-180.

39. Stadtman, E. R. 1952. The purification and properties of phosphotransacetylase. J. Biol. Chem. 164:769-793.

40. Stokstad, E. L. R., G. R. Seaman, R. J. Davis, and S. H. Hutner. 1955. Assay of thioctic acid, p. 23-27. *In* D. Glick (ed.), Methods of biochemical analysis, vol. 3. Interscience Publishing Co., New York.

41. Swartling, P. F. 1951. Biochemical and serological properties of some citric acid fermenting streptococci for milk and dairy products. J. Dairy Res. 18:256-267.

42. Thorne, K. I. J., and L. Kodreck. 1962. The metabolism of acetate and mevalonic acid by lactobacilli. I. The effect of acetate and mevalonic acid on growth. Biochim. Biophys. Acta 59:273-279.

43. Umbreit, W., R. W. Burris, and J. F. Stauffer. 1964. Manometric techniques, 4th ed. Burgess Publishing Co., Minneapolis.

44. Walton, E., A. F. Wagner, L. H. Peterson, F. W. Holly, and K. Folkers. 1954. The synthesis of (+) α-lipoic acid and its optical antipode. J. Amer. Chem. Soc. 76:4748.

45. Westerfeld, W. W. 1945. Colorimetric determination of blood acetoin. J. Biol. Chem. 161:495-502.

Utilization of Volatile Fatty Acids in Ruminants. III. Comparison of Mitochondrial Acyl Coenzyme A Synthetase Activity and Substrate Specificity in Different Tissues*

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ABSTRACT: Studies were undertaken to establish the important rate-limiting steps controlling acetate, propionate, and butyrate metabolism in ruminants. As an initial approach to this problem, the activity of acetyl, of propionyl, and of butyryl coenzyme A synthetase in mitochondria from various ruminant tissues was studied. Mitochondria were frozen and thawed three times to solubilize the enzymes. The acyl coenzyme A synthetase preparation was partially purified and its substrate specificity was studied. The results show that the activity of the enzyme active on acetate is high in highly aerobic tissues such as heart and in mammary gland and low in liver, in brain, in aorta, in skeletal muscle, and in rumen muscle. In heart, in mammary gland, in kidney, and in testes, the enzyme preparations were all about equally active on acetate and on propionate. In liver the enzyme preparation was equally active on propionate and on butyrate and least active on acetate. The enzyme from rumen epithelium was most active on butyrate, less active on propionate, and least active on acetate. Lung differs from all the other tissues in that the enzyme is very active on propionate and shows equal but lower activity for acetate and for butyrate. Marginal enzyme activity was found in skeletal muscle, in rumen muscle, in brain, and in aorta. The results offer an explanation for the preferential utilization of acetate by extrahepatic tissues, propionate by the liver, and butyrate by rumen epithelium. Butyrate, if present in peripheral blood, has a pharmacological effect on the pancreas. The ruminant has a unique mechanism for preventing butyrate appearance in peripheral blood, *i.e.*, a high butyryl coenzyme synthetase activity in rumen epithelium ensures rapid activation of butyrate for subsequent oxidation to β-hydroxybutyrate by the epithelium. If some butyrate escapes metabolism by the rumen epithelium, it is readily removed from portal blood by the liver where a high butyryl coenzyme A synthetase activity ensures rapid activation and subsequent metabolism to ketone bodies. Substrate specificity studies show that in addition to the three well known acyl coenzyme A synthetases active on straight-chain fatty acids (acid:ligase (adenosine monophosphate), EC 6.2.1.1, 6.2.1.2, and 6.2.1.3) there are at least three other acyl coenzyme A synthetases with distinct substrate specificities. The acyl coenzyme A synthetase from sheep liver is specific for C_1 to C_3 straight-chain fatty acids. The acyl coenzyme A synthetase from sheep kidney is specific for C_1 to C_3 straight-chain fatty acids. Sheep lung has an acyl coenzyme A synthetase specific for propionate. This work, along with earlier studies by others, would suggest that there are at least [illegible] coenzyme A synthetases in animal tissues with varying substrate specificities.

This laboratory is engaged in a study of control of acetate, of propionate, and of butyrate utilization in ruminants. These short-chain acids are produced in the rumen by microbial fermentation of carbohydrates and serve as primary metabolic substrates in ruminants. It has been shown that [illegible] normal conditions the ruminant liver is the [illegible] metabolizing propionate. Most of the acetate [illegible] blood is not removed by the liver and is [illegible] hepatic tissues ([illegible] Miller, [illegible]). [illegible] butyrate is not present in significant amounts [illegible] of portal blood (Cook and Miller, [illegible] 1967) and is believed to be oxidized to [illegible] absorption from the rumen (Ramsey and Davis, [illegible]).

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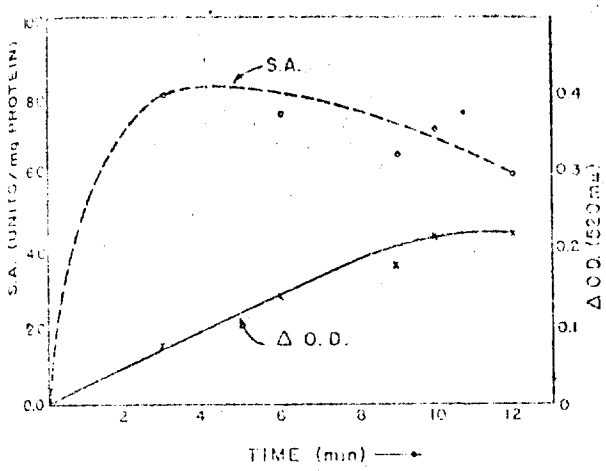

FIGURE 1: Effect of incubation time on the linearity of the acetyl-CoA synthetase reaction. The purified enzyme preparation from goat testicle was used for this study.

Preferential utilization of volatile fatty acids by ruminant tissues may be related to the tissue levels of fatty acid activating enzymes. Also, acyl-CoA synthetases are generally considered to be nonequilibrium enzymes and, therefore, can play a role in control of over-all cell processes. Consequently, the acetate, the propionate, and the butyrate activation reactions can function as important regulatory steps in short-chain fatty acid metabolism.

The purpose of the experiments reported in this communication was to study the activity of acetyl-, of propionyl- and of butyryl-CoA synthetase in ruminant tissues as a initial approach to the problem of elucidating control mechanisms regulating short chain fatty acid metabolism.

Experimental Section

Enzyme Assay. The acyl CoA synthetases were assayed by measuring the disappearance of the free sulfhydryl group of coenzyme A according to the procedure of Mahler *et al.* (1953). One enzyme unit is defined as 1 μmole of CoA reacted in 60 min. Specific activity is in units per milligram of protein. Protein was determined by the method of Lowry *et al.* (1951). A 10-min incubation time was chosen in order to measure a continuous change in optical density between control and enzyme-containing tubes (Figure 1). Enzyme activity was linear over the range of protein concentrations used in the reaction mixture for all tissues studied (an example is shown in Figure 2).

Isolation of Acetyl-CoA Synthetase. The method for isolation of mitochondria was similar to that reported by Hele (1954) for isolation of heart muscle mitochondria. The material sedimenting in the Sharples centrifuge is defined as the mitochondrial pellet. Although the purity of the mitochondrial pellet has not been clearly established, all ruminant tissues studied possess mitochondria including rumen epithelial cells as electron micrographs have demonstrated (Abumrad, Coppock, Clark and Spitler, 1967). The enzymes were extracted from the mitochondrial pellet by freezing and thawing and partially purified according to the procedure of Hele (1954).

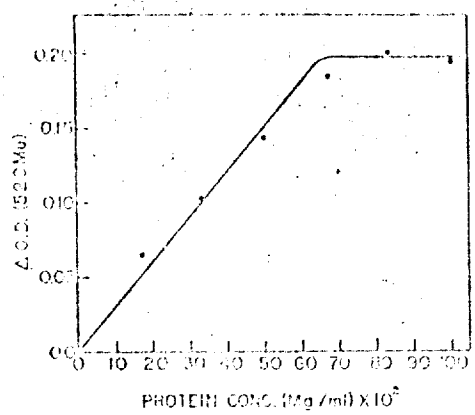

FIGURE 2: Effect of protein concentration on the linearity of the acetyl-CoA synthetase reaction. The enzyme from mammary gland was used for this study.

Results and Discussion

The activity of acetyl-, of propionyl-, and of butyryl-CoA synthetase was measured in the mitochondrial extract and purified preparations. Also, both the crude mitochondrial extracts and purified preparations were used for substrate specificity studies. The relative enzyme activities from both

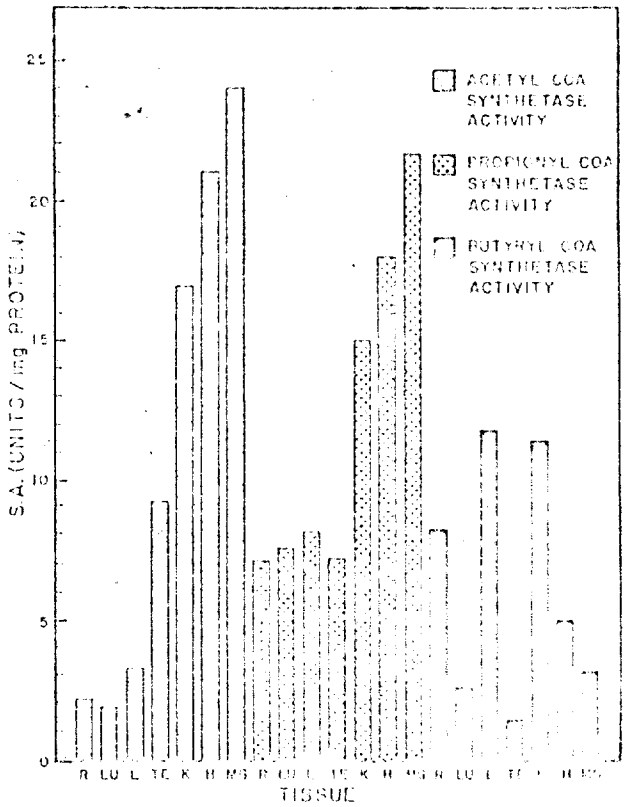

FIGURE 3: Summary of the activity of acetyl-, of propionyl-, and of butyryl-CoA synthetase from mitochondria of various ruminant tissues (R = rumen, LU = lung, L = liver, TE = testicle, K = kidney, H = heart, and MG = mammary gland).

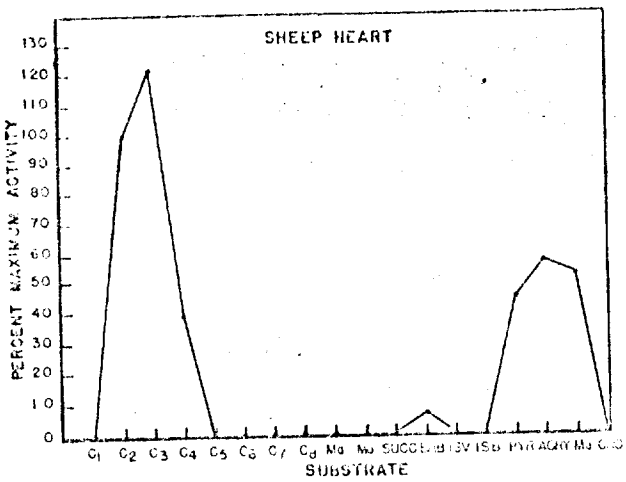

FIGURE 4: Substrate specificity of the enzyme preparation from sheep heart. The purest preparations from sheep heart as well as from mammary gland, from liver, from lung, from testes, and from kidney were used in the studies (Ma = malate, Mo = malonate, SUCC = succinate, BHB = betahydroxybutyrate, ISV = isovalerate, ISB = isobutyrate, PYR = pyruvate, ACRY = acrylate, Me = maleate, and CRO = crotonate).

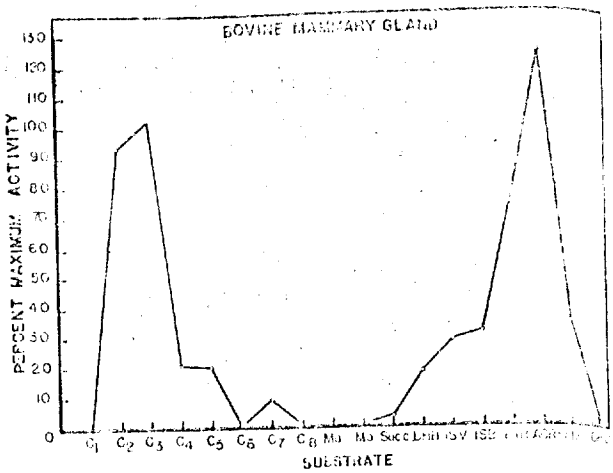

FIGURE 6: Substrate specificity of the enzyme preparation from bovine mammary gland.

fractions were similar. Studies using the purified enzyme preparations are reported.

Acetyl-CoA synthetase (Figure 3) is high in kidney, in heart, in mammary gland, and in testes but low in liver, in rumen epithelium, and in lung. However, all tissues have a very active propionyl-CoA synthetase. butyryl-CoA synthetase activity is high in rumen epithelium, in liver, and in kidney and is low in heart, in lung, in mammary gland, and in testes. With the exception of the enzyme isolated from lung, the enzyme preparations were either about equally active on acetate and on propionate and least active on butyrate or equally active on propionate and on butyrate and least active on acetate. Lung differs from all the other tissues in that the enzyme is specific for propionate.

Acetyl-, propionyl-, and butyryl-CoA synthetase activity was studied in brain, in skeletal muscle, in aorta, and in rumen muscle. Low levels of activity were found in aorta and in rumen muscle. Brain and skeletal muscle showed marked enzyme activity.

The active butyryl-CoA synthetase in rumen epithelium is consistent with studies showing that butyrate is readily metabolized by rumen epithelium (Stanton *et al.*, 1960; Kinsey and Davis, 1959). The oxidation of butyrate to β-hydroxybutyrate by rumen epithelium provides a mechanism for preventing the appearance of butyrate in peripheral blood. As shown by Phillips and Black (1966), butyrate in peripheral blood causes hyperglycemia by stimulating the release of glucagon from the pancreas. This pharmacological action of butyrate is not observed with acetate, with propionate, or β-hydroxybutyrate (R. W. Phillips, 1968, personal communication).

The activity of acetyl-CoA synthetase is consistent with *in vivo* studies showing that acetate is metabolized primarily by extrahepatic tissues. The absence of acetyl-CoA synthetase activity in skeletal muscle and the high activity in heart and in mammary gland suggests that acetate is metabolized

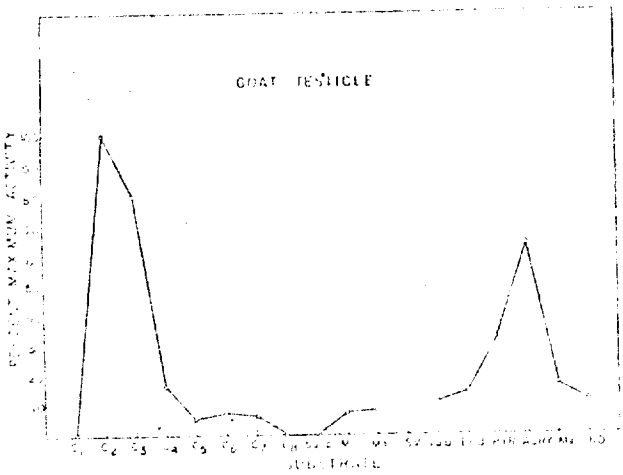

FIGURE 5: Substrate specificity of the enzyme preparation from goat testicle.

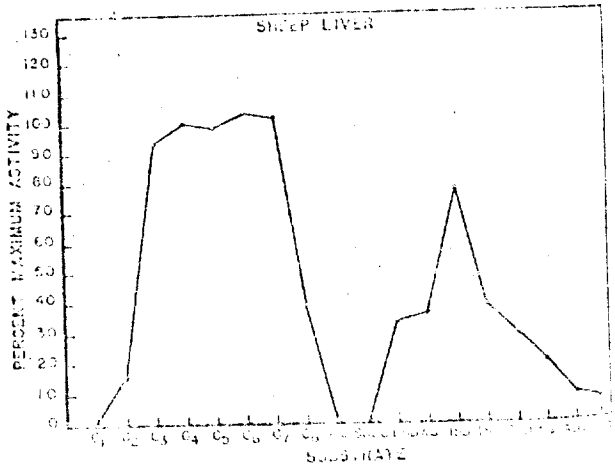

FIGURE 7: Substrate specificity of the enzyme preparation from sheep liver.

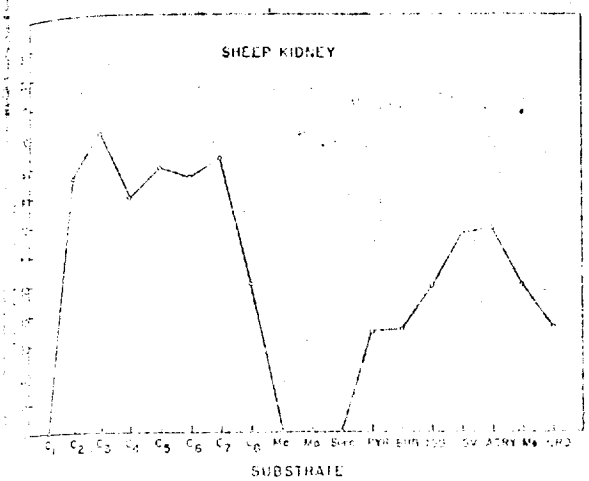

FIGURE 8: Substrate specificity of the enzyme preparation from sheep kidney.

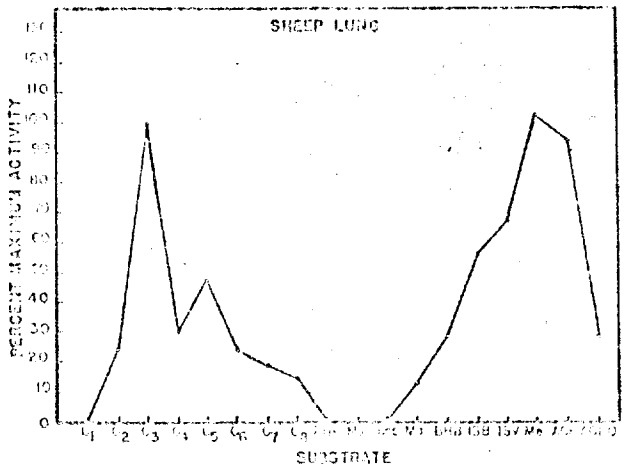

FIGURE 9: Substrate specificity of the enzyme preparation from sheep lung.

...ly by those extrahepatic tissues that are highly aerobic. ...studies have shown that propionate is removed from ...al blood by the liver under normal conditions (Cook and ...ller, 1965). However, when a high propionate fermentation ...roduced in the rumen, propionate is found in peripheral ...d and can be activated and metabolized by several tis... ...ucing kidney, lung, heart, and mammary gland.

...ubstrate specificity studies show the enzyme preparations ...heart, from mammary gland, and from testes to be spe... ...for acetate, propionate, and acrylate. The enzyme prep... ...tion from lung is specific for propionate and for acrylate. ...preparation from liver is specific for C_2 to C_3 straight-... ...in fatty acids, but the preparation from kidney is specific ...C_2 to C_3 fatty acids (Figures 4–9). It is commonly accepted ...there are three acyl-CoA synthetases in mammalian liver ...art. These enzymes are acetate:CoA ligase (AMP) (EC ...1.1) active on acetate propionate and acrylate; acid:CoA ...(AMP) (EC 6.2.1.2) active on C_3 to C_4 acids; acid:CoA ...(AMP) (EC 6.2.1.3) active on C_8 to C_{10} acids. The acyl-...A synthetases we have found in ruminant heart, in mam... ...gland, and in testes can be classified as acetate:CoA ...(AMP) (EC 6.2.1.1). However the acyl-CoA synthe... ...preparations from ruminant kidney, from lung, and from ...have not been previously reported. The liver, the lung, ...the kidney enzymes are three different enzymes and all ...are different from the heart, from the mammary gland, ...from the testes enzymes.

The data in this communication clearly show a genetic dif... ...between ruminant tissues in the activity of acyl-CoA ...thetases active on short-chain fatty acids. Although it is ...that many factors may be involved in the control ...metabolism, these results support our hypothesis ...activation reaction is an important control point in ...of short-chain fatty acid metabolism in ru... ...

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References

Annison, E. F., Hill, K. J., and Lewis, D. (1957), *Biochem. J.* 66, 592.

Black, A. L., Kleiber, M., and Brown, A. M. (1961), *J. Biol. Chem.* 236, 2399.

Cook, R. M., Lui, S.-C. C., and Wiese, A. C. (1964), *Fed. Proc.* 22, 134.

Cook, R. M., and Miller, L. D. (1965), *J. Dairy Sci.* 48, 1339.

Cook, R. M., and Wiese, A. C. (1966), *Fed. Proc.* 25, 544.

Hele, P. (1954), *J. Biol. Chem.* 206, 671.

Hyden, S., and Sperber, I. (1965), *in* Physical Digestion in the Ruminant, Dougherty, R. W., Ed., Washington, D. C., Butterworth, p 51.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* 193, 265.

Mahler, H. R., Wakil, S. J., and Bock, R. M. (1953), *J. Biol. Chem.* 204, 453.

Phillips, R. W., and Black, A. L. (1966), *J. Comp. Biochem. Physiol.* 18, 527.

Ramsey, H. A., and Davis, C. L. (1965), *J. Dairy Sci.* 48, 381.

Sutton, J. D., McGilliard, A. D., and Jacobson, N. L. (1963a), *J. Dairy Sci.* 46, 496.

Sutton, J. D., McGilliard, A. D., Richard, M., and Jacobson, N. L. (1963b), *J. Dairy Sci.* 46, 530.

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A Study of the Citric Acid Cycle in Certain Tumour Tissues

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At present there are two schools of thought about the respiration of cancer cells. Whereas both agree that tumour tissues exhibit high anaerobic and aerobic glycolysis with an excessive production of lactic acid (Cori & Cori, 1925; Warburg, 1926, 1956), they differ as to the extent of the operation of the citric acid cycle in these tissues (Weinhouse, 1955; Weinhouse, Warburg & Burk, 1956; Warburg, 1956). In these studies the activity of certain segments of the cycle has been determined by oxygen consumption and carbon dioxide production procedures (Warburg, 1926; Busch & Potter, 1952; Busch & Baltrush, 1954; Busch, 1955; Weinhouse, 1955; Warburg, 1956; Ibsen, Coe & McKee, 1958), by measuring the glycolytic and oxidative enzymes (Sibley & Lehninger, 1949; LePage, 1950; Meister, 1950; Potter, 1951; Weinhouse, 1951; Wenner, Spirtes & Weinhouse, 1952; Beck, 1958), and the cofactors of the electron-transport system (Dubois & Potter, 1942; Wenner & Weinhouse, 1953; Greenstein, Werne, Eschenbrenner & Leuthardt, 1944), or by following the utilization of the various intermediates of the Embden–Meyerhof scheme and the tricarboxylic acid cycle (Pardee & Potter, 1949; Elliott & Kalnitsky, 1950; Olson, 1951; Wenner & Weinhouse, 1952; Heidelberger, 1953; Busch & Baltrush, 1954). However, none of these investigators has utilized the simultaneous determination of the amounts and radioactivities of all the members of the citric acid cycle *in vitro* in the presence and absence of certain added labelled substrates and blocking agents. Such a procedure could reflect the activity of the cycle if an excess of substrate is present despite the fact that the individual cycle acids allegedly serve a 'catalytic' function. There are examples of variations in the amounts of the individual cycle acids under differing experimental conditions, for example, after the addition of specific blocking agents. The present study was designed to employ such a direct approach. The tumour and normal tissue preparations were incubated with [^{14}C]acetate,

[^{14}C]succinate or non-labelled malate and the amounts and activities of the individual cycle acids were determined as described by Dajani & Orten (1958). The results conclusively demonstrated a quantitatively reduced activity of the tricarboxylic acid cycle in the tumours as compared with the normal tissues studied under the conditions of the experiments.

EXPERIMENTAL

Materials

DPN, ATP, succinic acid and L-malic acid, all purified grade, were purchased from the California Foundation for Biochemical Research, Los Angeles, Calif. [1-^{14}C]Acetate and [1-^{14}C]succinate were obtained from Volk Radiochemical Co., Chicago, Ill. Penicillin G potassium was a product of Lederle Laboratories, New York; the dihydrostreptomycin was produced by Pfizer and Co., New York. Triethyltin acetate was kindly supplied by Dr K. E. Moore, University of Michigan, and by the Metal and Thermite Corp., New York City.

Three types of tumour tissues were used: Ehrlich mouse-ascites-tumour cells, and Walker and LC-18 carcinoma from adult rats. The Walker carcinoma was originally a human adenocarcinoma of the adrenals and the LC-18 carcinoma was originally a human hepatoma. Both had undergone repeated transfers in Fisher strain white rats and were implanted and grown subcutaneously. The normal tissues used for comparison were muscle, liver and isolated liver cells from Fisher strain adult rats and nucleated erythrocytes from Plymouth Rock chickens.

Procedure

Twenty per cent homogenates of pooled tumour and non-tumour tissues from 8–10 rats were prepared at 5° in 50 ml. of Krebs–Ringer phosphate buffer (pH 7·4) with the use of a Potter–Elvehjem glass homogenizer. To the homogenates were added the following substances, together with the various substrates used, dissolved in 5 ml. of Krebs–Ringer phosphate: L-malic acid, 0·05 mg.; ATP, 8·0 mg.; DPN, 2·0 mg.; monofluoroacetic acid (where used), 0·5 mg.; sodium malonate (where used), 75·0 mg.; penicillin, 1–2 mg.; streptomycin, 2–4 mg. Sodium fluoride (0·01M final concentration) was added to the Krebs–Ringer phosphate used in homogenization in order to prevent loss of ATP and DPN by adenosine triphosphatase and diphosphopyridine nucleotidase (NAD nucleosidase) respectively. In an additional series of experiments triethyltin acetate, a recently introduced powerful and complete adenosine-triphosphatase inhibitor (Dr K. E. Moore, personal communication), was added to the fluid mixture used in the homogenization of tissues in the recommended final concentration (1 μM). The supplementary DPN, ATP, as well as triethyltin acetate, were added as a further precautionary measure because there is some evidence that homogenized tissues, particularly certain tumours, exhibit increased activity of adenosine triphosphatase and probably diphosphopyridine nucleotidase. The amounts of the substrates used are given as subheadings to the appropriate Tables. The addition of these substrates to the tissue preparations followed the procedure described elsewhere (Dajani & Orten, 1958). The mixtures were incubated at 37–38° in $O_2 + CO_2$ (95:5) for 30 min. and 1, 4, 18 or 24 hr., with constant gentle shaking (250 oscillations/min.). Since incubation for the shorter intervals did not result in an accumulation of the cycle acids to significantly measurable quantities in the presence of inhibitors, the 4 hr. time interval was chosen for incubation in all subsequent experiments. Although a longer period, 18 hr., gave higher values, the picture obtained was similar and the shorter period was adopted. In all of these trial studies excess of substrate was always detected at the end of the experiment. However, after incubation for 24 hr. no substrate remained, and the levels of the cycle acids were diminished. It should be emphasized at this point that all the experimental conditions adopted were also found to support vigorous Krebs-cycle activity in nucleated erythrocytes (Dajani & Orten, 1958). As a further check, the pH of the mixtures was determined at the end of the 4 hr. period. No significant change had occurred. At the end of the incubation period, the enzymic reactions were stopped by freezing, and the extraction and determination of the citric acid-cycle acids and their ^{14}C-activities followed the procedure that has already been reported (Dajani & Orten, 1958). The citric acid-cycle acids were extracted with acid–acetone and the amounts and ^{14}C-activities of the individual cycle acids were quantitatively determined after separation by silica-gel-column chromatography by a procedure developed in this Laboratory and described in detail by Dajani & Orten (1958). Essentially the procedure involved the differential elution of the cycle acids from the silica-gel column by automatically increasing concentrations of *tert.*-amyl alcohol in chloroform. The eluted acids were collected in an automatic fraction collector and then titrated with standard sodium hydroxide by using a Rehburg microburette. The titrated fractions were then transferred to planchets, evaporated to dryness and the ^{14}C-activities determined in a gas-flow counter. The flow of effluent through the chromatographic column was slowed by the excessive amounts of lipid in the extracts of tumour tissue, resulting in a poor separation of the cycle acids, so the procedure was modified to remove lipid. For this purpose, the aqueous concentrate of the acid–acetone extract was shaken in a separating funnel with ether (10 ml.). After 15 min., the ether layer was transferred to aluminum planchets and evaporated to dryness, and the radioactivity was determined. The aqueous layer was used for determination of the cycle acids (Dajani & Orten, 1958). No significant loss of cycle acids by this treatment was found in recovery studies.

Preparation of cell suspensions. Ehrlich mouse-ascites-carcinoma fluid containing the tumour cells was aspirated from the peritoneal cavity of 20–25 ether-anaesthetized mice. The fluid was pooled and the free cells were obtained by differential centrifuging at 200*g* for 5 min. and then at 3000*g* for 15 min. The cells were washed twice with Krebs–Ringer phosphate and suspended in 50 ml. of Krebs–Ringer phosphate (pH 7·4), or Krebs–Ringer phosphate containing 20% of clear ascites fluid, to make a 20% cell suspension. Hepatic cells from normal rats were isolated by the procedure of Branster & Morton (1957). Nucleated erythrocytes were prepared as described by Dajani & Orten (1958). These cells were suspended in 50 ml. of Krebs–Ringer phosphate, to give a 20% suspension. The rest of the procedure was the same as that given above.

RESULTS

Experiments represented in Tables 1 and 2 on normal and tumour tissues permit a comparison of the utilization of [1-^{14}C]acetate by way of the Krebs cycle in these tissues. The amounts of acids listed in these and subsequent Tables are increases over controls (no substrate added). It is evident that the concentrations and ^{14}C-activities of the citric acid-cycle intermediates are lower in the tumour tissues than in normal liver or muscle. Also, there is more incorporation of ^{14}C into the cycle acids in LC-18 carcinoma tissue than in Walker carcinoma.

In the studies represented in Tables 1 and 2, it may be recalled that fluoride was added to the homogenates of the tissues to inhibit adenosine triphosphatase and diphosphopyridine nucleotidase. Since it appears possible that fluoride may not have completely inhibited these phosphatases when present at the elevated levels claimed in tumour tissues, triethyltin acetate was employed as an adenosine-triphosphatase inhibitor in place of fluoride. The results, given in Table 3, confirm those found when fluoride was used and thus indicate that the lower activity of the cycle, in LC-18 tumour tissues at least, is not due to a loss or destruction of ATP.

Table 1. *Average concentrations (μmoles/100 g.) of Krebs-cycle acids in tissues incubated with* [1-^{14}C]*acetate*

Values are net syntheses above controls and are averages of three experiments ± average deviations, with different batches of pooled tissues. The substrate contained: 0·45 mg. of [1-^{14}C]acetate (4·11 mc/m-mole); 4·0 mg. of sodium acetate trihydrate. These were dissolved in the incubation mixture, which contained in each 50 ml. of Krebs–Ringer phosphate buffer (pH 7·4): L-malic acid, 0·05 mg.; ATP, 8·0 mg.; DPN, 2·0 mg.; NaF, 21·0 mg.; penicillin, 2 mg.; dihydrostreptomycin, 4 mg.; monofluoroacetic acid (where used), 0·5 mg. No substrate was added to the controls.

Acid	Control	Acetate	Acetate + fluoroacetate	Control	Acetate	Acetate + fluoroacetate
		Liver			Muscle	
Citric	1·1 ± 0·02	3·0 ± 0·06	2·0 ± 0·02	1·0 ± 0·01	3·3 ± 0·03	4·6 ± 0·08
Aconitic (*cis* + *trans*)	0·4 ± 0·01	0·6 ± 0·02	0·9 ± 0·01	0·3 ± 0·01	0·5 ± 0·01	0·8 ± 0·02
*iso*Citric	0·5 ± 0·01	0·6 ± 0·01	0·8 ± 0·01	0·4 ± 0·02	0·7 ± 0·02	0·8 ± 0·01
α-Oxoglutaric	1·3 ± 0·03	3·8 ± 0·03	2·2 ± 0·02	0·9 ± 0·01	2·4 ± 0·03	2·1 ± 0·02
Succinic	1·7 ± 0·07	4·4 ± 0·07	3·8 ± 0·06	1·5 ± 0·03	4·2 ± 0·07	3·9 ± 0·06
Fumaric	1·5 ± 0·06	3·8 ± 0·05	3·6 ± 0·05	1·4 ± 0·02	3·9 ± 0·05	3·7 ± 0·03
L-Malic	1·3 ± 0·07	3·5 ± 0·06	3·3 ± 0·03	1·3 ± 0·04	3·6 ± 0·05	3·5 ± 0·07
Oxaloacetic + acetoacetic	0·2 ± 0·01	1·0 ± 0·02	6·2 ± 0·07	0·3 ± 0·01	0·8 ± 0·02	5·9 ± 0·06
		Walker carcinoma			LC-18 carcinoma	
Citric	0·5 ± 0·01	0·7 ± 0·02	1·5 ± 0·03	0·5 ± 0·07	0·6 ± 0·03	1·7 ± 0·06
Aconitic (*cis* + *trans*)	0·2 ± 0·03	0·5 ± 0·04	0·9 ± 0·02	0·3 ± 0·02	0·5 ± 0·04	1·1 ± 0·03
*iso*Citric	0·3 ± 0·03	0·5 ± 0·03	0·9 ± 0·01	0·3 ± 0·04	0·6 ± 0·02	1·1 ± 0·01
α-Oxoglutaric	0·4 ± 0·03	1·5 ± 0·06	1·4 ± 0·06	0·3 ± 0·03	1·7 ± 0·03	1·5 ± 0·03
Succinic	0·2 ± 0·01	1·4 ± 0·04	1·0 ± 0·04	0·6 ± 0·02	2·1 ± 0·05	1·8 ± 0·08
Fumaric	0·3 ± 0·04	1·4 ± 0·03	1·0 ± 0·03	1·5 ± 0·06	2·4 ± 0·05	1·9 ± 0·07
L-Malic	0·3 ± 0·03	1·4 ± 0·02	0·9 ± 0·04	0·4 ± 0·04	1·2 ± 0·04	1·2 ± 0·03
Oxaloacetic + acetoacetic	0·4 ± 0·02	1·3 ± 0·02	2·9 ± 0·06	0·3 ± 0·03	1·3 ± 0·03	3·8 ± 0·03

Table 2. *Average* ^{14}C *counts* (10^{-3} × *counts/min./100 g.*) *of Krebs-cycle acids in tissues incubated with* [1-^{14}C]*acetate*

Values are averages of three experiments, with different batches of pooled tissues. The substrate and composition of the incubation mixture are given in Table 1.

Acid	Liver		Muscle		Walker carcinoma		LC-18 carcinoma	
	Acetate	Acetate + fluoroacetate	Acetate	Acetate + fluoroacetate	Acetate	Acetate + fluoroacetate	Acetate	Acetate + fluoroacetate
Citric	110·0	195·0	125·0	203·0	7·4	8·7	7·9	9·5
Aconitic (*cis* + *trans*)	30·0	31·0	38·0	30·0	3·2	2·7	3·4	2·8
*iso*Citric	38·0	32·0	36·0	34·0	3·2	2·6	3·3	2·7
α-Oxoglutaric	95·0	61·0	68·0	59·0	4·3	4·0	4·8	4·2
Succinic	125·0	96·0	122·0	115·0	2·2	2·1	2·9	2·4
Fumaric	104·0	100·0	113·0	110·0	2·1	2·0	3·2	2·5
L-Malic	92·0	87·0	109·0	107·0	2·0	1·8	3·6	2·0
Oxaloacetic + acetoacetic	27·0	217·0	22·0	163·0	4·5	8·4	4·6	12·0

Table 3. *Average concentration and ^{14}C-activity of Krebs-cycle acids in tissue homogenates incubated with [1-^{14}C]acetate in the presence of triethyltin acetate*

Values are net syntheses above controls and are averages of two experiments with different batches of pooled tissues. The compositions of the substrate and incubation mixture are given in Table 1, with the exception that triethyltin acetate at a final concentration of 1 μM replaced NaF as an adenosine-triphosphatase inhibitor.

Acid	Liver (μmoles/100 g.)	Liver (10^{-3} × Counts/min./100 g.)	LC-18 carcinoma (μmoles/100 g.)	LC-18 carcinoma (10^{-3} × Counts/min./100 g.)
Citric	3·2 ± 0·02	112·0	0·7 ± 0·02	8·1
Aconitic (*cis* + *trans*)	0·7 ± 0·02	33·0	0·4 ± 0·03	3·1
*iso*Citric	0·6 ± 0·03	35·0	0·5 ± 0·03	3·3
α-Oxoglutaric	4·1 ± 0·05	100·0	1·8 ± 0·04	4·0
Succinic	4·3 ± 0·02	122·0	2·0 ± 0·05	3·0
Fumaric	4·0 ± 0·06	108·0	2·3 ± 0·04	3·3
L-Malic	3·6 ± 0·05	94·0	1·3 ± 0·07	3·8
Oxaloacetic + acetoacetic	1·2 ± 0·03	30·0	1·4 ± 0·03	4·9

Table 4. *Cycle acids in ascites-tumour cells incubated with [1-^{14}C]acetate + 20% of ascites fluid*

Values are net syntheses above controls and are averages of three experiments, ± average deviations, with different batches of pooled tissues. The compositions of the substrate and the incubation mixture are given in Table 1. Where used, 75·0 mg. of sodium malonate was added to the incubation mixture. No substrate was added to the controls. Blank values indicate amounts too small to be measured accurately.

Acid	μmoles/100 g. of packed cells: Control	Acetate	Fluoroacetate added	Malonate added	10^{-3} Counts/min./100 g. of packed cells: Acetate	Fluoroacetate added	Malonate added
Citric	—	1·5 ± 0·03	2·3 ± 0·05	2·1 ± 0·06	4·3	6·4	6·0
Aconitic (*cis* + *trans*)	—	—	—	—	1·4	1·3	1·6
*iso*Citric	—	—	—	—	1·4	1·2	1·6
α-Oxoglutaric	—	1·3 ± 0·02	1·0 ± 0·03	1·2 ± 0·02	3·3	2·9	3·4
Succinic	0·9 ± 0·03	2·0 ± 0·04	1·8 ± 0·05	2·4 ± 0·03	5·4	5·3	6·9
Fumaric	0·7 ± 0·02	1·9 ± 0·05	1·8 ± 0·07	1·1 ± 0·01	5·1	4·9	4·3
L-Malic	—	1·6 ± 0·04	1·5 ± 0·03	1·5 ± 0·05	4·2	3·9	3·9
Oxaloacetic + acetoacetic	—	2·7 ± 0·06	3·3 ± 0·07	2·4 ± 0·08	7·5	9·4	6·9

The amounts of lactic acid and pyruvic acid in the different tissues were also determined together with the cycle acids in all experiments. The concentrations of these two acids were invariably greater in all tumour tissues studied than in the normal tissues. This observation is in agreement with that of other investigators (Olsen, 1951; Heidelberger, 1953; Warburg, 1956; Weinhouse *et al.* 1956).

Because the Walker and LC-18 tumours are a composite tissue and have a varying cancer-cell content, their use could, undoubtedly, lead to uncertainty in interpretation. It was thought advisable, therefore, to carry out further experiments on Ehrlich mouse-ascites-tumour cells, which are a nearly pure culture of intact cancer cells. Moreover, the utilization of unruptured ascites cells minimizes the uncertainty of ATP loss resulting from homogenization. At first, suspensions of these cells in Krebs–Ringer phosphate alone were used. The results of three experiments on different preparations of ascites cells indicated that acetate was incorporated into the various citric acid-cycle acids to a lesser degree than that in the normal tissues. Most of the substrate appeared to have been diverted mainly to the formation of acetoacetate and fatty acids. Indeed, a greater portion of the ^{14}C-activity resided in the ether extract mentioned above. In further experiments, in order to provide more nearly physiological conditions for incubation, 20% of ascites fluid was incorporated into the incubation medium, replacing an equivalent amount of Krebs–Ringer phosphate. The use of ascites fluid in the incubation medium has been found advantageous by others (Kun, Talalay & Williams-Ashman, 1951; Warburg, 1956). Indeed, in the present study, the inclusion of ascites fluid in the incubation mixture employed in this and the subsequent experiments increased the concentrations and activities of the citric acid-cycle acids over those obtained with Krebs–Ringer phosphate alone. As is evident from the data presented in Table 4, however, the activity of the cycle is less than that of the normal tissues studied (Tables 1 and 2).

Since high concentrations of DPN have been reported to increase the oxygen uptake of hepatoma and liver mitochondria (Wenner & Weinhouse, 1952), the effect of the two concentrations of this cofactor (2 or 5 mg.) on the utilization of acetate by the ascites-tumour cells was studied. In spite of the fact that the addition of DPN did not seem to raise the concentrations and activities of the Krebs-cycle intermediates to any appreciable extent, its inclusion in the incubation mixture, as stated earlier, was continued simply as a precautionary measure.

In addition to the effect of fluoroacetate upon the activity of the Krebs cycle, malonate, a specific inhibitor of succinic dehydrogenase (Pardee & Potter, 1949), was also studied. Its use as a blocking agent should result in the accumulation of succinate. The results in Table 4 show that, although there was an accumulation of succinate, this was small. As with fluoroacetate inhibition, this observation points to a reduced activity of the cycle in these cells. This is not in complete disagreement with the experiments *in vivo* of other investigators (Busch & Potter, 1952), who demonstrated that several tissues, including five tumours, accumulated succinate in the presence of malonate. Their data were interpreted to mean only that the Krebs cycle is functioning in these tissues, and do not point to the extent of the cycle activity. Despite this finding, however, these authors cautioned against drawing definite conclusions based on their data alone, since succinate may have arisen from sources other than the citric acid-cycle intermediates, e.g. glutamic acid.

The results given thus far strengthen the thesis that the citric acid cycle is operating at a lower level in the tumour tissues studied than in normal tissues. This could be due to a block(s) of an unknown nature either within or outside the cycle, hindering acetate from entering into the cycle. In order to obtain further evidence on the former point, two of the intermediates of the cycle itself were employed as substrates, in addition to acetate.

In one series, non-labelled L-malic acid mixed with labelled acetate was incubated with the tumour-cell suspension. In another, radioactive succinate mixed with non-labelled acetate was used. The results of these experiments are given in Tables 5 and 6 respectively. Again, the data clearly demonstrated that these two intermediates were not utilized by the cycle enzymes better than was acetate alone (Table 4). This was expected, at least with respect to succinate, since the succinic-dehydrogenase activity in tumour tissue is believed to be low (Schneider & Potter, 1943). Further, the occurrence of a weak electron-transport chain reported by Schneider & Potter (1943) and others (Greenstein *et al.* 1944) could also be a contributing factor. The use of fluoroacetate and malonate as inhibitors, also, did not significantly alter the concentrations of citrate or succinate respectively. This finding is similar to that obtained above when acetate alone was used as a substrate (Table 4). If there were an impairment in the pathway between acetate and citrate, one of two possibilities, or both, could be a contributing factor(s). Either the activating-enzyme system of acetate which brings about the formation of acetyl-CoA or the condensing enzyme might not be functioning normally. Other studies, however, appear to rule out the latter possibility (Wenner *et al.* 1952). This leaves the former as a possible alternative mechanism. One could speculate, therefore, that acetyl-CoA may not have been biosynthesized in sufficient quantities or that its production was at such a slow rate that it could not keep pace with the turnover by the condensing enzyme. To explore this point, the systems of the experiments that follow were fortified with 5 mg. of CoA to test its effect on the utilization of acetate or succinate by

Table 5. *Cycle acids in ascites-tumour cells incubated with substrate containing* 1·5 *mg. of* L-*malic acid*

Values are net syntheses above controls and are averages of three experiments, ±average deviations, with different preparations of ascites cells. The compositions of the substrate and incubation mixture are given in Tables 1 and 4. Blank values indicate amounts too small to be measured accurately.

	μmoles/100 g. of packed cells				10^{-3} × Counts/min./100 g. of packed cells		
Acid	Control	Acetate + malate	Fluoro-acetate added	Malonate added	Acetate + malate	Fluoro-acetate added	Malonate added
Citric	—	1·4±0·02	2·3±0·07	2·3±0·07	4·5	6·5	6·1
Aconitic (*cis* + *trans*)	—	—	—	—	1·3	1·3	1·4
*iso*Citric	—	—	—	—	1·4	1·2	1·4
α-Oxoglutaric	—	1·3±0·03	1·2±0·08	1·5±0·04	3·4	3·2	3·3
Succinic	0·8±0·03	2·3±0·04	2·0±0·1	2·5±0·08	5·3	5·3	6·3
Fumaric	0·7±0·02	2·2±0·05	1·9±0·04	1·7±0·09	5·0	4·9	4·4
L-Malic	—	1·9±0·04	1·7±0·05	2·0±0·06	4·5	4·1	4·1
Oxaloacetic + acetoacetic	—	3·0±0·08	3·4±0·06	3·3±0·06	7·8	9·7	6·9

Table 6. *Cycle acids in ascites-tumour cells incubated with* [1-^{14}C]*succinic acid*

Values are net syntheses above controls and are averages of two experiments, ±average deviations, with different preparations of ascites cells. The substrate contained: succinic acid, 5·0 mg.; [1-^{14}C]succinic acid, 1·5 mg. (activity 1·3 mc/m-mole); sodium acetate trihydrate, 2·0 mg.; in 50 ml. of Krebs–Ringer phosphate. The composition of the incubation mixture is given in Table 1. Blank values indicate amounts too small to be measured accurately. The high counts for succinic acid are due primarily to non-utilized [1-^{14}C]succinic acid.

Acid	μmoles/100 g. of packed cells			10^{-3} × Counts/min./100 g of packed cells	
	Control	Succinate	Succinate + fluoroacetate	Succinate	Succinate + fluoroacetate
Citric	—	1·3±0·03	1·4±0·02	11·5	12·0
Aconitic (*cis* + *trans*)	—	—	—	5·8	4·7
*iso*Citric	—	—	—	5·3	4·0
α-Oxoglutaric	—	0·8±0·01	0·7±0·03	7·5	7·8
Succinic	0·9±0·02	20·8±0·08	21·0±0·04	361·5	395·0
Fumaric	0·8±0·02	1·7±0·04	1·6±0·06	18·8	18·0
L-Malic	—	1·6±0·07	1·7±0·05	17·4	16·8
Oxaloacetic + acetoacetic	—	1·1±0·03	1·2±0·06	14·0	14·5

Table 7. *Cycle acids in ascites-tumour cells incubated with substrate containing acetyl-coenzyme A*

Values are net syntheses above controls and are from one experiment. The compositions of the substrate and incubation mixture are given in Table 1, with the exception that 2 μmoles of acetyl-CoA were added. No substrate was added to the controls. Blank values indicate amounts too small to be measured accurately.

Acid	μmoles/100 g. of packed cells		10^{-3} × Counts/min./100 g. of packed cells.
	Control	Acetate + acetyl-CoA + fluoroacetate added	Acetate + acetyl-CoA + fluoroacetate added
Citric	—	2·3	6·4
Aconitic (*cis* + *trans*)	—	—	1·3
*iso*Citric	—	—	1·2
α-Oxoglutaric	—	1·2	2·9
Succinic	0·9	2·0	5·3
Fumaric	0·7	1·9	4·9
L-Malic	—	1·5	3·9
Oxaloacetic + acetoacetic	—	3·4	9·3

way of the citric acid cycle in ascites cells. If CoA is lacking or not present in adequate amounts, its addition to the incubation medium would be expected to raise the concentrations of the cycle members, provided that the other cofactors and the activating enzyme are not only present but are also functioning normally. It is assumed, of course, that this would be the case if the cycle itself is also operating at a normal level. When it was found that CoA failed to increase the concentrations of the cycle acids significantly under the experimental conditions, a further investigation with preformed acetyl-CoA was pursued (Table 7) in an effort to determine whether or not the activating system itself might be defective. Contrary to expectation, no increase in citrate in the presence of fluoroacetate was observed (cf. Table 4), a finding that can be considered as indirect evidence against an impairment of the acetate-activating system. The only increase under these conditions was in the oxaloacetate–acetoacetate fraction. This observation will be discussed later.

To assess still further the operation of the citric acid cycle in ascites-tumour cells, the activity of the cycle was compared under identical conditions with that in isolated liver cells and in nucleated erythrocytes (also suspensions of intact cells). The data (Table 8) show that the quantities and activities of the cycle members biosynthesized in hepatic cells and in nucleated erythrocytes are several times greater than those found in ascites-tumour cells. Particularly striking is the accumulation of citrate in nucleated erythrocytes when fluoroacetate is added. Likewise, when malonate is added to hepatic cells, there is a marked accumulation of succinate.

Table 8. *Comparison of cycle acids in ascites-tumour cells, isolated hepatic cells and nucleated erythrocytes incubated with the same substrate*

Values are net syntheses above controls and are averages of three experiments, ± average deviations, with different preparations of pooled cells. The compositions of the substrate and incubation mixture are given in Tables 1 and 4. Blank values indicate amounts too small to be measured accurately. The values for nucleated erythrocytes are taken from Dajani & Orten (1958).

Acid	μmoles/100 g. of packed cells			10^{-3} × counts/min./100 g. of packed cells		
	Acetate	Fluoroacetate added	Malonate added	Acetate	Fluoroacetate added	Malonate added
			Ascites cells			
Citric	1·5±0·03	2·3±0·05	2·1±0·06	4·3	6·4	6·0
Aconitic (*cis* + *trans*)	—	—	—	1·4	1·3	1·6
*iso*Citric	—	—	—	1·4	1·2	1·6
α-Oxoglutaric	1·3±0·02	1·0±0·03	1·2±0·02	3·3	2·9	3·4
Succinic	2·0±0·04	1·8±0·05	2·4±0·03	5·4	5·3	6·9
Fumaric	1·9±0·05	1·8±0·07	1·1±0·01	5·1	4·9	4·3
L-Malic	1·6±0·04	1·5±0·03	1·5±0·05	4·2	3·9	3·9
Oxaloacetic + acetoacetic	2·7±0·06	3·3±0·07	2·4±0·08	7·5	9·4	6·9
			Isolated hepatic cells			
Citric	3·0±0·02	1·9±0·03	1·9±0·05	113·0	112·0	110·5
Aconitic (*cis* + *trans*)	0·7±0·01	0·6±0·04	0·6±0·04	30·5	29·5	32·5
*iso*Citric	0·8±0·02	0·5±0·02	0·5±0·02	38·0	34·0	35·0
α-Oxoglutaric	4·2±0·04	2·6±0·03	4·5±0·05	97·0	63·0	102·0
Succinic	4·3±0·03	3·9±0·05	6·7±0·06	128·0	99·0	165·5
Fumaric	3·8±0·06	3·6±0·06	2·1±0·03	107·0	101·0	75·8
L-Malic	3·3±0·06	3·2±0·04	2·2±0·04	93·0	88·0	66·0
Oxaloacetic + acetoacetic	1·2±0·02	5·8±0·05	3·5±0·07	29·0	212·0	97·0
			Nucleated erythrocytes			
Citric	28·5	49·5		1350·0	2310·0	
Aconitic (*cis* + *trans*)	1·6	1·1		67·2	48·5	
*iso*Citric	1·6	1·1		68·8	47·9	
α-Oxoglutaric	16·0	3·6		432·0	95·5	
Succinic	49·7	48·5		1650·0	1740·0	
Fumaric	51·0	49·5		1440·0	1390·0	
L-Malic	39·5	39·0		1220·0	1080·0	
Oxaloacetic + acetoacetic	28·0	135·0		785·0	3780·0	

DISCUSSION

It appears from work in other laboratories (see references cited in introduction), with manometric enzymic and isotopic techniques, that segments of the citric acid cycle proceed, to some extent at least, in tumour tissue. On the other hand, there is also evidence which indicates that the activity of the cycle is of a lower order than that of many normal tissues. The results presented in the present paper, however, indicate clearly that the activity of the citric acid cycle in the tumour tissues studied is of a lower order than that in the normal control tissues. This is based on the interpretation that the concentrations and total ^{14}C-activities of the intermediates in the cycle reflect the amount of material passing through it. Another possible interpretation, on the other hand, is that the lower concentrations of the acids in the tumour tissues could mean that the cycle is as active or even more active in these tissues, provided that the enzyme levels are higher. However, measurement of the levels of certain enzymes of the citric acid cycle and some other related enzymes, have shown that they are either comparable or lower, but never higher, in tumour tissues. The enzymes measured include cytochrome oxidase (Greenstein *et al.* 1944), succinic dehydrogenase and cytochrome oxidase (Schneider & Potter, 1943), aconitase (Weinhouse, 1951), malic dehydrogenase (Potter, 1946), condensing enzyme (Potter & Busch, 1950), and lactic, α-oxoglutaric, malic and *iso*citric dehydrogenases, fumarase, oxaloacetic carboxylase, condensing enzyme and aconitase (Wenner *et al.* 1952). Further, an equal or higher activity of the cycle in tumour tissues would require a higher rate of acetate utilization when acetate was used as the substrate. Actually, acetate utilization has been found by several investigators to be much less in tumour than in the normal tissues that they have studied. Pardee, Heidelberger & Potter (1950) measured the oxidation of [1-^{14}C]acetate to $^{14}CO_2$ in slices and homogenates of tumour and non-tumour tissues and found that less than one-

twenty-fifth as much $^{14}CO_2$ was produced by tumour as by kidney. Similar results were later obtained by Busch & Baltrush (1954). Another line of evidence for the occurrence of a reduced activity of the Krebs cycle was provided by Kit & Greenberg (1951), who were able to show that acetate was oxidized to carbon dioxide in Gardner lymphosarcoma to about one-third the extent of that in spleen, one of the normal tissues of relatively low oxidizing activity. Also, citrate accumulation was almost of the same magnitude in the presence of fluoroacetate as in its absence. Weinhouse (1951), on the other hand, has shown that $^{14}CO_2$ is formed in approximately equal quantities from various substrates, including acetate, by tumour and normal-tissue slices. Further, when [2-^{14}C]pyruvate is used as a substrate, $^{14}CO_2$ production in hepatoma was nearly the same as that produced in liver (Olson, 1951). Thus most of the observations *in vitro* cited above indicate a lower activity of the cycle in the tumour tissues studied, as was found by a different procedure in the present study.

Further evidence of a diminished activity of segments of the citric acid cycle have been furnished by several experiments *in vivo*. Potter & Busch (1950) determined the citric acid content of tissues of fluoroacetate-treated rats and found it to accumulate in most tissues with the exception of liver, testis and tumour. The inability of the liver, which is known to exhibit high citric acid-cycle activity, to accumulate citrate was explained on the basis of the occurrence of another pathway for acetate and pyruvate utilization, namely acetoacetate formation. The absence of citrate accumulation in tumour was considered as 'strong evidence that the ability of tumour tissues to form citrate is very low in comparison with a wide variety of normal tissues'. Other experiments on transplanted rat tumour *in vivo* also showed either no significant acetate or pyruvate oxidation (Busch & Baltrush, 1954; Busch, 1955), or, with the uptake of glutamate or succinate, activity of only a portion of the citric acid cycle (Nyhan & Busch, 1958).

Recently Busch, Davis & Olle (1957) have demonstrated that only 'under optimal conditions (i.e. excess of a variety of substrates of the citric acid cycle, pH 7·4, and an atmosphere of 100 per cent oxygen) is the capacity of transplantable rat tumour for production of citrate essentially the same as that of a variety of non-tumour tissues'. Even under such unphysiological conditions as these, their data show that citrate accumulation was significantly greater in the normal tissues than in any of the tumours investigated.

The present study provides a more complete and direct demonstration of a low activity of the entire Krebs cycle in certain tumours. It is evident that, with the exception of the oxaloacetate fraction, which contains an appreciable quantity of acetoacetate, the amounts and activities (Tables 1 and 2) of all the cycle intermediates in liver or muscle were always greater than in the two tumour tissues. The experiments with fluoroacetate furnished further support for this observation. The apparent increase in the oxaloacetate fraction would be expected from the effect of fluoroacetate inhibition of the citric acid cycle and the diversion of a considerable portion of acetate towards fatty acid biosynthesis (Crandall, Gurin & Wilson, 1947).

The studies of Ehrlich ascites cells gave even more convincing evidence about the status of the cycle in tumours. Here again, the quantities and activities of the cycle acids (Table 4) are decidedly less than those in isolated liver cells or nucleated erythrocytes (Table 8). Further, the values are less than those for liver or muscle (Tables 1 and 2). These differences are also apparent if specific activities, which reflect rate of turnover and hence the extent of the activity of the cycle enzymes, are calculated and then compared. Indeed, the specific activities of the individual intermediates in tumours are definitely much less than their counterparts in the normal tissues.

Although the foregoing observations tend to support the view that the Krebs cycle is of low activity in tumours, they have left undecided the question of whether or not a block(s) exists outside the cycle which could hinder the normal entrance of acetate into it. One could assume either an enzyme or a cofactor, or both, to be the limiting factor(s). As a matter of fact, several cofactors, such as DPN, ATP and CoA, are known to be involved in these reactions and would retard the rate of citrate biosynthesis if they were not present in sufficient quantities. Also, two enzyme systems are recognized as being concerned with the reactions that lead to citrate formation from acetate, namely the activating and condensing enzymes. The latter, however, is not believed to exist in tumours in levels comparable with those in non-tumours (Wenner *et al.* 1952). Because DPN and ATP were used in all experiments, including the control, only the CoA was tested for its effect on the utilization of acetate or succinate by way of the citric acid cycle in the ascites cells. The present results make it clear that the cycle components did not increase any more in the presence of CoA than in its absence. This would suggest that CoA either does not penetrate the cell readily or that tumours are less able to activate acetate than many normal tissues. Although no attempt was made to investigate the permeability of the ascites cell to CoA in the present investigation, the formation of acetyl-CoA was studied by indirect means. If acetate

were not normally metabolized via the citric acid cycle because of a lack or a subnormal activity of the activating enzyme, it would be reasonable to assume that adding preformed acetyl-CoA would increase the output of citrate in the presence of fluoroacetate. Acetyl-CoA did not produce any such increase in the citrate concentration (Table 3). It is, of course, possible that acetyl-CoA may also not have penetrated the cell or that it may have been deacylated. The simplest explanation of the inactivity of acetyl-CoA is, of course, that it does not penetrate the cell. However, if it be assumed that permeability and deacylation are not in fact important factors, then these results suggest that there is no deficiency of activating-enzyme activity in ascites-tumour cells and perhaps in other types of tumours. This is supported by the observation that there was a marked increase in the production of acetoacetate and fatty acids; such a finding argues against an impairment in the activating-enzyme system, since acetyl-CoA formation is established to be the first step in the biosynthesis of these substances. This finding would also argue against any possible excessive destruction of ATP and DPN, which, of course, could retard the cycle activity in the tumour tissues employed.

In view of the present findings, together with those in the literature cited, it seems reasonable to conclude that the reactions of the Krebs cycle are of a lower order in the tumour as compared with normal tissues investigated. A diminished amount or activity, or both, of some intracycle enzyme system(s) appears to be the limiting factor.

SUMMARY

1. The operation of the Krebs cycle in certain tumour and non-tumour tissues has been studied by measuring the concentrations and ^{14}C-activities of the cycle intermediates when acetate, malate and/or succinate were used as substrates in the presence and absence of the blocking agents, fluoroacetate and malonate. The utilization of these substances by way of the citric acid cycle was less in the Walker, LC-18 and mouse ascites tumours than in muscle, liver (homogenates and isolated intact cells) or nucleated erythrocytes.

2. Addition of adenosine triphosphate and two concentrations of diphosphopyridine nucleotide to the incubation mixture, in the presence of fluoride or triethyltin acetate, did not result in any appreciable increase in the amounts or ^{14}C-activities of the citric acid-cycle members in tumours. Likewise, fortifying the incubation mixture with either coenzyme A or preformed acetyl-coenzyme A failed to produce a significant increase in the quantities and activities of the cycle acids. The only increase was in the lipid and acetoacetate fractions. This was considered to indicate that there was no apparent impairment in the activating-enzyme system or excessive loss of adenosine triphosphate or diphosphopyridine nucleotide in the tumours employed.

3. The cycle intermediates, succinate and malate, were not utilized any better by the tumour tissues than was acetate.

4. In view of the above findings, it is concluded that the lower activity of the cycle in the tumour than in the normal tissues studied is primarily due to some defect in an intra-Krebs-cycle reaction(s).

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REFERENCES

Beck, W. S. (1958). *J. biol. Chem.* **232**, 251.
Branster, V. M. & Morton, R. K. (1957). *Nature, Lond.*, **180**, 1283.
Busch, H. (1955). *Cancer Res.* **15**, 365.
Busch, H. & Baltrush, H. A. (1954). *Cancer. Res.* **14**, 448.
Busch, H., Davis, J. R. & Olle, E. W. (1957). *Cancer Res.* **17**, 711.
Busch, H. & Potter, V. R. (1952). *Cancer Res.* **12**, 660.
Cori, C. F. & Cori, G. T. (1925). *J. biol. Chem.* **65**, 397.
Crandall, D. I., Gurin, S. & Wilson, D. W. (1947). *Fed. Proc.* **6**, 246.
Dajani, R. M. & Orten, J. M. (1958). *J. biol. Chem.* **231**, 913.
Dubois, K. P. & Potter, V. R. (1942). *Cancer Res.* **2**, 290.
Elliott, W. B. & Kalnitsky, G. (1950). *J. biol. Chem.* **186**, 487.
Greenstein, J. P., Werne, J., Eschenbrenner, A. B. & Leuthardt, F. M. (1944). *J. nat. Cancer Inst.* **5**, 55.
Heidelberger, C. (1953). *Advanc. Cancer Res.* **1**, 273.
Ibsen, K. H., Coe, E. L. & McKee, R. W. (1958). *Biochim. biophys. Acta*, **30**, 384.
Kit, S. & Greenberg, D. M. (1951). *Cancer Res.* **11**, 495.
Kun, E., Talalay, P. & Williams-Ashman, H. G. (1951). *Cancer Res.* **11**, 835.
LePage, G. A. (1950). *Cancer Res.* **10**, 77.
Meister, A. (1950). *J. nat. Cancer Inst.* **10**, 1263.
Nyhan, W. L. & Busch, H. (1958). *Cancer Res.* **18**, 385, 1203.
Olsen, R. E. (1951). *Cancer Res.* **11**, 571.
Pardee, A. B., Heidelberger, C. & Potter, V. R. (1950). *J. biol. Chem.* **186**, 625.
Pardee, A. B. & Potter, V. R. (1949). *J. biol. Chem.* **178**, 241.
Potter, V. R. (1946). *J. biol. Chem.* **165**, 311.
Potter, V. R. (1951). *Cancer Res.* **11**, 565.

Potter, V. R. & Busch, H. (1953). *Cancer Res.* **10**, 353.
Schneider, W. C. & Potter, V. R. (1943). *Cancer Res.* **3**, 353.
Sibley, J. A. & Lehninger, A. L. (1949). *J. nat. Cancer Inst.* **9**, 393.
Warburg, O. (1926). *Über den Stoffwechsel der Tumoren.* Berlin: Julius Springer.
Warburg, O. (1956). *Science*, **123**, 309.
Weinhouse, S. (1951). *Cancer Res.* **11**, 585.
Weinhouse, S. (1955). *Advanc. Cancer Res.* **3**, 269.
Weinhouse, S., Warburg, O. & Burk, D. (1956). *Science*, **124**, 267.
Wenner, C. E., Spirtes, M. A. & Weinhouse, S. (1952). *Cancer Res.* **12**, 44.
Wenner, C. E. & Weinhouse, S. (1952). *Cancer Res.* **12**, 306.
Wenner, C. E. & Weinhouse, S. (1953). *Cancer Res.* **13**, 21.

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STIMULATION OF GASTRIC MOTILITY BY ACID

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In the course of experiments on the permeability of the gastric mucosa,[1-4] separated (Heidenhain) pouches of the dog's oxyntic gland area were frequently observed to contract vigorously when they were irrigated with 100 mM acetic acid or with a solution of 20 mM acetylsalicylic acid (aspirin) in 100 mM HCl. The normal gastric mucosa contains a barrier which prevents H^+ from diffusing rapidly into it from the gastric lumen,[5, 6] but acetic acid, because it is fat soluble, diffuses into the gastric mucosa about 3 times more rapidly than does HCl at a comparable concentration.[1, 6, 7] Salicylates, diffusing into the mucosa from solutions in which they are un-ionized and therefore fat soluble,[2, 8] break the mucosal barrier to H^+ with the consequence that the mucosa becomes more permeable to HCl concurrently or subsequently applied to it. The high incidence of strong contractions occurring in circumstances when acid was penetrating the mucosal barrier suggested that acid itself might stimulate motility, and, accordingly, the experiments reported in this paper were planned.

Methods

Three dogs with separated (Heidenhain) pouches of the oxyntic gland area of the stomach were used. The animals were in good health, accustomed to the laboratory, and trained to stand quietly with very light restraint throughout the tests. The opening of the pouch on the abdominal wall was fitted with a vitallium cannula which did not leak. Each pouch could be filled with more than 40 ml of fluid without the animal showing signs of discomfort. Before each test, the dogs were deprived of food but not of water for 18 to 20 hr.

The cannula was directly connected by polyethylene tubing with the head of a Statham strain gauge, the output of which was recorded with a Grass polygraph. By means of T-valves, the pouch could be filled or emptied and the gauge could be calibrated with a mercury manometer. Zero pressure level was that of the opening of the pouch on the abdominal wall. Three indexes of motility were used. (1) The *area* under the pressure time curve above 15 mm Hg was measured with a planimeter; the units are millimeters of mercury minutes for the period over which the record was measured. (2) The *fraction* of time that the pressure was greater than 15 mm Hg was measured; it is dimensionless. (3) The *frequency* of contractions generating more than 15 mm Hg pressure was determined; its units are contractions for the period noted.

Five solutions were used in the first part of the study: (1) 250 mM glycine, *p*H, 6 to 7; (2) 100 mM HCl; (3) 100 mM acetic acid; (4) 20 mM acetylsalicylic acid in 100 mM HCl; and (5) 20 mM acetylsalicylic acid in 1 mM HCl, *p*H, 2.7 to 3.7. These solutions contained 15 mM NaCl and were made isotonic with appropriate concentrations of mannitol. All solutions were brought to 39 C before being infused, and the standard volume of 40 ml was used in each test.

Results

In 25 experiments on separate days over a period of 3 months, the pouch of dog 1 was filled as gently as possible with 40 ml of glycine solution, and pressure recording was immediately begun. At the end of 1 hr, the pouch was drained and refilled with one of the five solutions listed above, and pressure was recorded for a 2nd hr. Then the pouch was drained and refilled with 40 ml of the glycine solution, and pressure was recorded for a 3rd hr.

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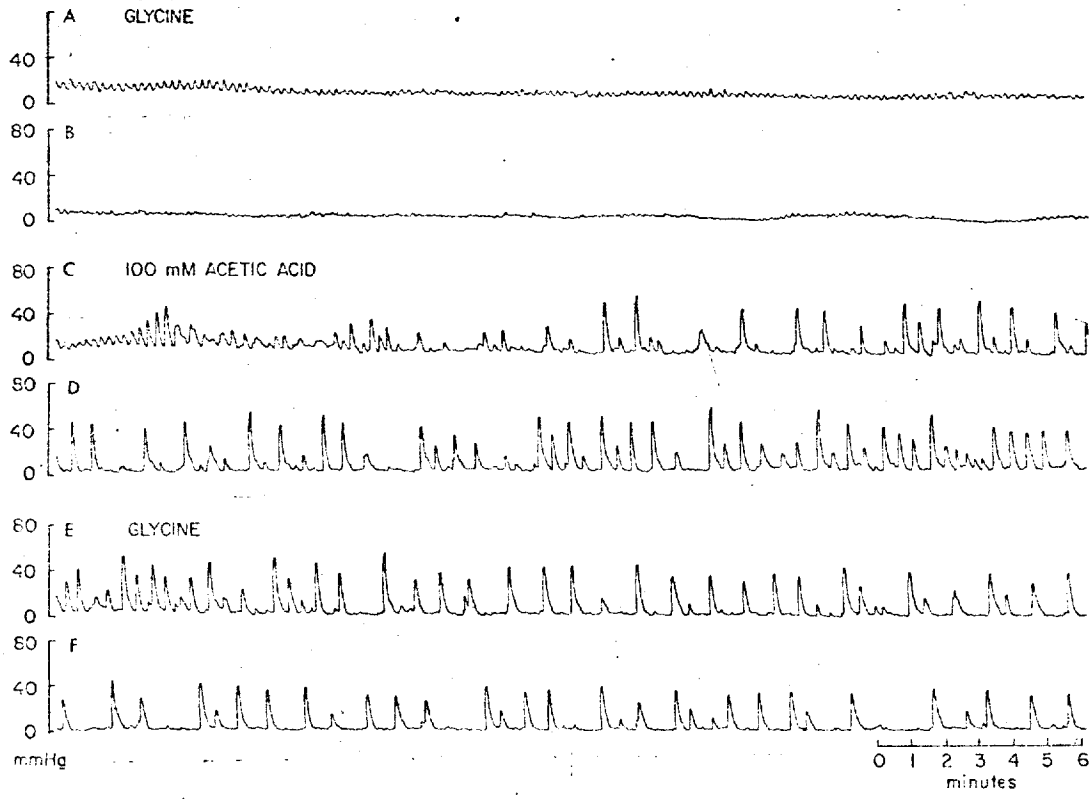

FIG. 1. Pressure recorded from pouch of dog 1 when the pouch was filled with 40 ml of 250 mM glycine solution (*A* and *B*), then with 100 mM acetic acid (*C* and *D*), and finally with glycine solution again (*E* and *F*). Each pair of traces is a continuous record obtained over 1 hr, and there were approximately 3-min intervals between *B* and *C* and between *D* and *E*.

The record obtained in one of the five experiments in which the pouch was filled during the 2nd hr with 100 mM acetic acid is shown in figure 1. Traces *A* and *B* show the low degree of motility observed in 24 of the 25 instances in which the pouch was filled with the glycine solution. Traces *C* and *D* show that, immediately after the pouch was filled with acetic acid, motility increased to a high level, and this continued unabated even when the pouch was refilled with glycine solution. The same response occurred in each of the other four acetic acid experiments.

The record obtained in one of the five experiments in which the pouch was filled in the second period with 20 mM acetylsalicylic acid in 100 mM HCl is shown in figure 2. About 20 min after this solution was placed in the pouch, strong contractions appeared and continued for the rest of the experiment. Results of other experiments differed only in that strong contractions began earlier after introduction of 20 mM aspirin in 100 mM HCl.

There was no increase in motility in any of the five experiments in which 20 mM aspirin in 1 mM HCl was placed in the pouch. There was, in fact, less motility than was recorded when the pouch was filled only with glycine solution. Evidence that aspirin itself reduces the excitability of the oxyntic gland area is presented below.

Figure 3 summarizes the results. The *top bar* of each triplet represents the mean area ± SD for the 1st hr of five experiments in which the pouch was filled with glycine solution. The *second bar* represents the mean area ± SD when the pouch was filled with the solution named on the *left*. The *third bar* represents the mean

FIG. 2. Pressure recorded from pouch of dog 1 when the pouch was filled with 250 mM glycine solution (*A* and *B*), then with 20 mM aspirin in 100 mM HCl (*C* and *D*), and finally with glycine solution again (*E* and *F*). Each pair of traces is a continuous record obtained over 1 hr, and there were approximately 3-min intervals between *B* and *C* and between *D* and *E*.

area ± SD when the pouch was finally filled with glycine solution. The *P* values denote significance of difference of means from the means of the corresponding period in the glycine experiments. The same pattern of significance of differences was obtained when frequencies or fractions were compared.

Motility did not increase in any period in which the pouch was filled with 100 mM HCl alone. However, in each instance after this solution was removed and the pouch was filled with glycine solution, motility promptly increased and remained at a high level for 60 min. Since the data given below do not show that 100 mM HCl increased excitability of the pouch, there is no immediately obvious explanation for this behavior.

In each of 2 other dogs, the separated pouch of the oxyntic gland area was filled with 40 ml of one of the five solutions, pressure was recorded for 1 hr, and the day's observations were terminated. The observations with glycine were repeated 15 times and those with the other solutions five times each. Areas are given in table 1. Fraction and frequency which showed the same pattern of significance are omitted for the sake of economy. The results confirm those obtained on dog 1 in the following respects. In these dogs, filling the pouch with 100 mM acetic acid invariably stimulated vigorous and sustained motility; filling the pouch with glycine solution, 100 mM HCl alone, or 20 mM acetylsalicylic acid in 1 mM HCl did not.

The results differed from those obtained in dog 1 in that 20 mM aspirin in 100 mM HCl did not stimulate contractions. This

failure might be attributed to a depressing effect of aspirin upon excitability the depression being able to block stimulation by HCl which the presence of aspirin permitted to diffuse into the mucosa. Proof of this explanation requires two kinds of evidence: that HCl actually could stimulate in dogs 2 and 3, and that acetylsalicylic acid could be shown to inhibit motility.

The rate at which HCl diffuses into the mucosa can be increased by the simple expedient of raising its luminal concentration. Since the permeability coefficient of the mammalian gastric mucosa for acetic acid is almost exactly 3 times as large as that for HCl,[9] filling the pouches with 200 to 300 mM HCl should produce the same effect as filling with 100 mM acetic acid.

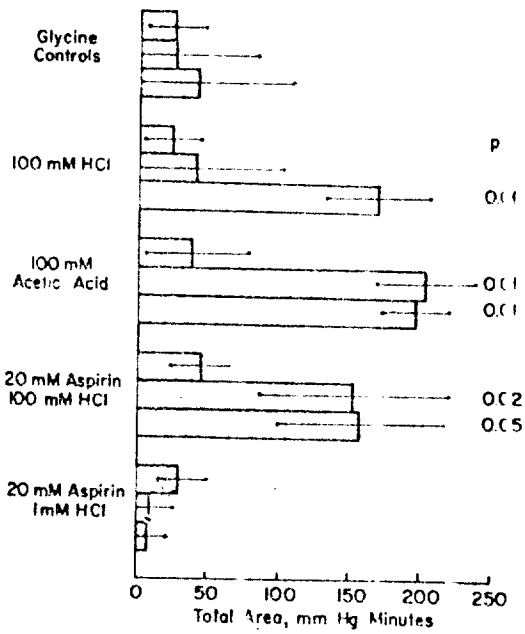

FIG. 3. Summary of results obtained in five groups of 3-hr experiments on dog 1 with five replications in each group. *Top bar* of a triplet represents mean ± SD of the area under the pressure time curve above 15 mm Hg when the pouch was filled for the 1st hr with 250 mM glycine solution. *Second bar* represents mean ± SD when the pouch was filled for the 2nd hr with the solution named on the *left*. *Third bar* represents mean ± SD when the pouch was filled for the 3rd hr with glycine solution again. The *P* values denote significance of difference of means from corresponding hour in glycine experiments.

TABLE 1. *Index of motility of separated pouches of the oxyntic gland area of the stomach of 2 dogs*

Solution[a]	Dog	n[b]	Area[c]
			mm Hg min/hr
Glycine	2	15	51 ± 43
	3	15	37 ± 34
100 mM HCl	2	5	29 ± 24
	3	5	44 ± 59
100 mM acetic acid	2	5	237 ± 66[d]
	3	5	132 ± 21[d]
20 mM aspirin in 100 mM HCl	2	5	23 ± 38
	3	5	35 ± 44
20 mM aspirin in 1 mM HCl	2	5	21 ± 28
	3	5	42 ± 54

[a] Pouches filled with 40 ml of solution indicated; motility recorded for 1 hr. Exact composition of solutions given under "Methods."

[b] Number of observations.

[c] Area under pressure time curve above 15 mm Hg; means ± SD.

[d] Significantly greater than corresponding glycine control, $P < 0.01$.

Preliminary experiments showed that 200 mM HCl did not consistently stimulate; therefore, the pouches of dogs 2 and 3 were filled with 250 mM HCl. As controls, the pouches were filled on different days with 250 mM NaCl. It was originally intended to record for 30 min. The characteristic effect of acetic acid invariably occurred within 10 min (trace C, fig. 1), and a comparable effect of HCl should be apparent equally soon. In addition, the short period of observation decreased the possibility of stimulation by increasing volume within the pouch, the increase being caused either by fluid output which follows damage[4] or by the osmotic pressure gradient across the mucosa.[10] This schedule could not be followed with dog 2, for, on four of the nine occasions on which her pouch was filled with 250 mM HCl, the experiment had to be terminated before 30 min because the dog retched and vomited. Consequently, these abbreviated periods were matched with NaCl control periods of the same length performed on subsequent days. The time given for dog 2 in table 2 is the mean length of the nine

TABLE 2. *Index of mot[illegible] of separated pouches of the oxyntic gl[illegible] area of 2 dogs*

Dog	Solution	Duration	n^a	Area[b]
		min		
2	250 mM NaCl	25	9	31 ± 20
	250 mM HCl	25	9	76 ± 29[c]
3	250 mM NaCl	30	10	25 ± 31
	250 mM HCl	30	10	55 ± 27[d]

[a] *n*, Number of observations; 40 ml in pouches.

[b] Area under pressure time curve above 15 mm Hg; units are millimeters of mercury minutes for time indicated, mean ± SD.

[c] Significantly greater than corresponding NaCl value, $P < 0.01$.

[d] Significantly greater than corresponding NaCl value, $P < 0.05$.

periods. Areas are given; fractions and frequencies displayed the same pattern of significance. It is clear that HCl could stimulate in these dog[illegible]

In order to determine whether acetylsalicylic acid itself inhibits gastric motility, it is necessary to establish an experimental situation in which the drug is able to enter the mucosa in the absence of a high concentration of acid and to test the ability of the neuromuscular apparatus of the stomach to respond to an innocuous stimulus. It has already been shown that salicylate diffuses into the gastric mucosa as rapidly from a solution of 1 mM HCl as from 100 mM HCl.[2] Accordingly, inhibition of motility, if it occurs, should be demonstrable when 20 mM aspirin in 1 mM HCl is in the pouch. After preliminary trials, the following protocol was devised. A T-tube connected to a 50-ml syringe was inserted between the cannula and the strain gauge. The pouch was gently filled with 40 ml of the test solution, and recording was begun. Exactly 30 min later, when any slight motility stimulated by simple filling of the pouch had died away, the dog was given 0.4 mg of bethanechol chloride subcutaneously. Then 30 min after injection, 40 ml of fluid were withdrawn by means of the syringe and reinfused, 5 sec being taken for withdrawal and 5 for reinfusion. Wi[illegible]awal and reinfusion were repeated f[illegible] total of five times over a period of 5[illegible]. The purpose of the bethanechol inj[illegible]n was to establish an intermediate [illegible]l of excitability in the pouch which [illegible]a be depressed or enhanced, and the pu[illegible]e of the withdrawal and infusion was [illegible]ive a standard mechanical stimulus whi[illegible]would test excitability. The 0.4-mg [illegible] of the drug was found to be just the [illegible]t one; smaller doses did not raise excit[illegible]ity sufficiently to permit mechanical sti[illegible]tion to evoke consistent responses, an[illegible]ger doses in themselves stimulated me[illegible]y.

The experiments were [illegible]formed nine times each with the gl[illegible] solution, 20 mM aspirin in 1 mM HCl, [illegible]M HCl alone, and 100 mM HCl. Dog [illegible] was used, for studies on dogs 2 and 3 [illegible]ved that their response to mechanical s[illegible]lation was so irregular that an unco[illegible]nable number of experiments would have been required to establish significant differences. The use of the solutions was rotated according to a predetermined schedule, so that any spontaneous changes occurring during the 2 months occupied by the tests would have a random effect upon the results. The area under the pressure time curve was measured for the 30 min between bethanechol injection and withdrawal infusion. Likewise, fractions and frequencies were determined. The same indexes of motility were also determined for the 15 min following withdrawal infusion. The means ± SD are given in table 3. There were no detectable differences among the results obtained when the pouch was filled with glycine, 1 mM HCl, or 100 mM HCl; these solutions neither enhanced nor depressed excitability. It is clear, however, that filling the pouch with a solution from which aspirin is rapidly absorbed depressed motility and excitability. Lim and his colleagues[11] have presented cogent evidence that aspirin and sodium salicylate act peripherally by blocking impulse generation by pain receptors. One can surmise that a similar peripheral blocking action was responsible for aspirin's effect on pouch motility.

TABLE 3. *Indexes of motility of a separated pouch of the oxyntic gland area of a dog's stomach after cholinergic and mechanical stimulation*[a]

	Area[b]	Fraction[c]	Contractions[d]
30 min after bethanechol[e]			
Glycine	19 ± 21	0.077 ± 0.074	28 ± 25
Aspirin in 1 mM HCl	5 ± 12	0.020 ± 0.040	9 ± 19
1 mM HCl	25 ± 21	0.069 ± 0.054	27 ± 20
100 mM HCl	16 ± 14	0.048 ± 0.047	19 ± 15
15 min after withdrawal infusion			
Glycine	28 ± 17	0.173 ± 0.077	21 ± 9
Aspirin in 1 mM HCl	3 ± 7[f]	0.029 ± 0.055[f]	5 ± 9[f]
1 mM HCl	22 ± 16	0.121 ± 0.077	20 ± 12
100 mM HCl	26 ± 20	0.113 ± 0.082	18 ± 9

[a] Means ± SD; *n*, nine in each category.

[b] Area under pressure time curve above 15 mm Hg for period indicated; units are millimeters of mercury minutes.

[c] Fraction of time during period indicated pressure was above 15 mm Hg; no units.

[d] Total number of contractions generating pressure greater than 15 mm Hg during period indicated.

[e] See text for description of experimental protocol and composition of solutions.

[f] Significantly less than corresponding values obtained with other solutions, $P < 0.01$.

Discussion

Hydrochloric acid placed in the duodenum arouses vigorous local contractions,[12] but 100 mM HCl in contact with the oxyntic gland area does not. Acid rapidly diffuses through the duodenal mucosa, whereas it is prevented from rapidly diffusing into the gastric mucosa by a barrier. If acid is able to diffuse rapidly through the gastric barrier because it is fat soluble (acetic acid), because it has a high luminal concentration (250 mM HCl), or because the barrier is broken (100 mM HCl plus aspirin), then gastric motility may be stimulated. Afferent fibers in vagal nerves which have endings in the stomach that are stimulated by acid have been identified.[13] Similar sensory neurons may be present in the intramural plexuses, and they may be the afferent link in a reflex chain by which gastric motility is stimulated.

Summary

Motility of separated (Heidenhain) pouches of the oxyntic gland area of 3 dogs' stomachs was measured when the pouches were filled with 250 mM glycine solution (pH, 6 to 7), 100 mM HCl, 100 mM acetic acid, 20 mM acetylsalicylic acid (aspirin) in 100 mM HCl, or 20 mM acetylsalicylic acid in 1 mM HCl. In all dogs, acetic acid invariably stimulated vigorous contractions. In 1 dog, 20 mM acetylsalicylic acid in 100 mM HCl also stimulated motility. Failure of this solution to stimulate motility in the other dogs was attributed to the depressing effect of acetylsalicylic acid itself. That HCl could stimulate in these dogs was demonstrated by raising its luminal concentration to 250 mM. Depression of gastric motility and excitability was demonstrated in experiments in which excitability was elevated by bethanechol injection and motility was mechanically stimulated. It is concluded that acid, when it can diffuse rapidly into the mucosa, stimulates motility.

REFERENCES

1. Davenport, H. W. 1964. Gastric mucosal injury by fatty and acetylsalicylic acids. Gastroenterology *46:* 245–253.
2. Davenport, H. W. 1965. Damage to the gastric mucosa: effects of salicylates and stimulation. Gastroenterology *49:* 189–196.
3. Davenport, H. W. 1965. Potassium fluxes across the resting and stimulated gastric mucosa: injury by salicylic and acetic acids. Gastroenterology *49:* 238–245

4. Davenport, H. W. 1966. Fluid produced by the gastric mucosa during damage by acetic and salicylic acids. Gastroenterology *50:* 487–499.
5. Code, C. F., J. A. Higgins, J. C. Moll, A. L. Orvis, and J. F. Scholer. 1963. The influence of acid on the gastric absorption of water, sodium, and potassium. J. Physiol. (London) *166:* 110–119.
6. Davenport, H. W., H. A. Warner, and C. F. Code. 1964. Functional significance of gastric mucosal barrier to sodium. Gastroenterology *47:* 142–152.
7. Teorell, T. 1939. On the permeability of the stomach for acids and some other substances. J. Gen. Physiol. *23:* 263–274.
8. Schanker, L. S. 1960. On the mechanism of absorption of drugs from the gastrointestinal tract. J. Med. Pharm. Chem. *2:* 343–359.
9. Flenström, G., B. Frenning, and K. J. Öbrink. 1964. The disappearance of acetic acid and acetate from the cat's stomach and its influence on the permeability for hydrochloric acid. Acta Physiol. Scand. *62:* 422–428.
10. Moody, F. G., and R. P. Durbin. 1965. Effects of glycine and other instillates on the concentration of gastric acid. Amer. J. Physiol. *209:* 122–126.
11. Lim, R. K. S., F. Guzman, D. W. Rodgers, K. Goto, G. D. Dickerson, and R. J. Engle. 1964. Site of action of narcotic and nonnarcotic analgesics determined by blocking bradykinin-evoked pain. Arch. Int. Pharmacodyn. *152:* 25–56.
12. Brink, B. M., J. F. Schlegel, and C. F. Code. 1965. The pressure profile of the gastroduodenal junctional zone in dogs. Gut *6:* 163–171.
13. Iggo, A. 1957. Gastric mucosal chemoreceptors with vagal afferent fibres in the cat. Quart. J. Exp. Physiol. *42:* 398–409.

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A SURVEY OF CHEMICALS FOR MUTAGENIC ACTION ON E. COLI[1]

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INTRODUCTION

At present we have available two good methods, using *Escherichia coli*, for the quantitative study of spontaneous mutability and of the mutagenic action of various agents such as radiations and chemicals. The older of the two methods deals with mutational changes from phage sensitivity to phage resistance, and the more recently developed one (Demerec, 1951; Bertani, 1951) with back-mutations from streptomycin dependence to nondependence. In both cases the mutants can be easily classified; very large numbers of bacteria may be tested; and slight increases in mutation rate can be easily detected.

After the streptomycin method had been developed we undertook extensive tests of a number of chemicals for mutagenicity, using mainly this method because it is the simpler of the two.

SELECTION OF CHEMICALS

Previous work by Witkin with *E. coli* (Witkin, 1947; Demerec, Wallace, and Witkin, 1948) had indicated that compounds exhibiting quite different properties may all be mutagenic. Using the phage method, she found that sodium desoxycholate, pyronin, acriflavine, caffeine, and colchicine acted as mutagens, but detected no mutagenic effect in tests with methyl green, basic fuchsin, and methylcholanthrene endosuccinate.

In planning the present survey, therefore, we prepared a list of chemicals, including representatives of widely separated groups, from simple inorganic and organic compounds to complex organic chemicals. Before the scheduled tests had been completed, however, we found that certain ferrous and manganous salts are very potent mutagens, and our attention was thus diverted to a detailed study of these compounds. Since it will require some time to

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carry this out, the data so far available from the survey are being summarized here.

MATERIALS AND METHODS

The "streptomycin" method for detection of induced mutations has been fully described by Bertani (1951). Only technical details will be given here.

Strains B/Sd-4/1,3,4,5 and B/Sd-4/3,4 of *E. Coli* were used throughout this work. For every experiment, bacteria were grown for 24 hours at 37°C in an aerated broth culture containing 10 micrograms of streptomycin per milliliter. They reached a saturation titer of approximately 2 to 3 × 10^9 cells per milliliter. Each culture was started from an inoculum, usually large, taken from streptomycin-agar slants kept in a refrigerator. Before treatment the bacteria were washed in saline and resuspended in distilled water. A sample of the new suspension was added to the desired solution of chemical in distilled water, and incubated at 37°C for a certain period of time; no growth occurs under these conditions. Another sample of the same suspension was added to an equal amount of distilled water and incubated for the same period of time, as a control. At the end of the treatment period, both treated and control suspensions were assayed by plating suitable dilutions on streptomycin-agar plates. At the same time they were plated (0.1 ml per Petri dish), either undiluted or diluted not more than 1:10 in plain broth, onto a number of streptomycin-free plates, using a glass spreader and a turntable. The assay plates were incubated for 48 hours, after which it was possible to count the colonies and calculate the titers of the two suspensions at the end of treatment, and the percentage of survivors. The streptomycin-free plates (mutant plates) were incubated for at least six days. After this time the colonies were scored, and the frequency of mutants calculated by dividing the number of colonies by the number of (viable) bacteria plated.

The length of the period of incubation in distilled water does not appreciably affect the proportion of mutations in the controls. In all but a few exceptional cases, nevertheless, the control suspensions were incubated for the same period of time as the chemically treated suspensions.

The degree of crowding of the dependent bacteria on the mutant plates would be expected to affect the observed frequency of mutants, as it influences the extent of residual growth and thus the chance that a mutant cell will develop into a visible colony. This effect is slight, however, when the bacteria plated have been raised in 10 micrograms of streptomycin per milliliter.

The chemical treatment also may limit the extent of residual growth, causing a spurious lowering of the frequency of mutants in the treated series. This may explain why, in experiments with some chemicals, the treated series showed frequencies lower than those of the controls.

Several of the chemicals used cause the formation of clumps of bacteria during treatment. If these clumps have not dissolved by the time the treated suspension is assayed to determine survival, the observed survival value

will be too low, and consequently the calculated mutation frequency too high. Therefore, except in the very first experiments done (*i.e.*, those with sulfuric, nitric, and hydrochloric acids, which in any case were not found to be mutagenic), the treated suspensions were always examined under the microscope before plating, and discarded if clumping was detected. By varying the concentration of chemical and the length of treatment, it was possible to eliminate clumping in many cases.

In a few scattered experiments (and in most of those done with phenol and formaldehyde) the dependent bacteria were grown in 25 instead of 10 micrograms of streptomycin per milliliter. It is known that this results in greater residual growth and therefore a larger number of spontaneous mutants per plate. This explains why in some experiments the spontaneous mutant frequencies were higher than average.

RESULTS

The results of a number of experiments for each chemical are reported in table 1. The concentration of the chemical shown in column 2 is the effective concentration at the time of treatment (*i.e.*, corrected for the addition of the bacterial suspension in distilled water). Number of plates (column 5) is the number of streptomycin-free plates used for the scoring of mutants. Total number of bacteria (column 6) is the number of viable dependent bacteria put on each plate, multiplied by the number of plates used. Number of mutants per 10^8 bacteria (column 7) is the mutant frequency, calculated by dividing the total number of colonies scored in an experiment by the total number of bacteria. The total number of mutant colonies is not given in the table, but can easily be calculated by the inverse operation. From it, one can determine the average number of mutants per plate, which in the experiments reported here was rarely larger than 10–20. Assuming a Poisson distribution, the variance can be estimated and the statistical significance of the results evaluated. Sometimes the same controls were common to several treated series; and in these cases the number of plates and total number of bacteria are reported only once for the controls. Different experiments done with each chemical are listed in order of decreasing survival.

Inorganic acids. Tests made with sulfuric, orthophosphoric, nitric, and hydrochloric acids gave negative results. Under certain conditions, all four of these compounds produced clumping of the treated bacteria, and this explains why in some experiments the values obtained for mutant frequency were higher in the treated series than in the controls. Negative results for hydrochloric acid had also been obtained in earlier experiments with Drosophila (Goldat and Beliaieva, 1935).

Boric acid was the only inorganic acid tested that gave positive results. In all experiments but one, the treated series showed an increase in mutation frequency. No clumping was ever observed among bacteria treated with boric acid.

Alkalies. Sodium hydroxide and potassium hydroxide were tested, with inconclusive results. Under the conditions of these experiments they never produced clumping.

Ammonia. Ammonia had been used in experiments with Drosophila by Lobashov and Smirnov (1934) and Lobashov (1937), and found to have mutagenic action. In the tests reported here, ammonia showed definite mutagenic activity in experiments where the proportion of survivors was lower than 2 per cent. Apparently similar positive results were obtained with ammonium chloride; they are not reported here, however, because clumping could not be avoided after treatment with this chemical.

Hydrogen peroxide. Attention was first drawn to the mutagenic activity of peroxides by the work of Stone, Wyss, and Haas (1947) on the indirect effect of mutation-inducing radiations on bacteria. Dickey, Cleland, and Lotz (1949), using Neurospora, studied the mutagenic potency of hydrogen peroxide and of a number of organic peroxides, and obtained positive results. In our experiments also, hydrogen peroxide was clearly shown to be mutagenic.

Metal salts. Mercuric chloride, silver nitrate, copper sulfate, and sodium silicate were tested. Only copper sulfate showed indications of mutagenic effect in the lower range of survival. Mutagenic potency had already been demonstrated for this salt by Magrzhikovskaja (1936) and Law (1938) in experiments with Drosophila. The results reported for mercuric chloride and silver nitrate do not exclude the possibility of their having mutagenic activity at the lowest survival levels, because it was extremely difficult to obtain survivals below 1.0 per cent and yet not so low as to fall beyond the range of sensitivity of the method.

Organic acids. Lobashov and Smirnov (1934) and Lobashov (1937) had treated Drosophila with acetic acid, but failed to detect any mutagenic activity of this substance. In our experiments, acetic, formic, and lactic acids were tested. The first two showed mutagenic activity. Some of the experiments with lactic acid indicated a weak mutagenic effect; but this is rendered doubtful by the fact that experiments with sodium lactate consistently gave negative results.

Formaldehyde. The mutagenic potency of this substance had been discovered by Rapoport (1946) and confirmed by W. D. Kaplan (1948) and Auerbach (1949), working with Drosophila. Clear-cut positive results were obtained in our experiments also. Auerbach's hypothesis that production of mutations by formaldehyde can occur only through some intermediate effect on substances contained in Drosophila food is not supported by our experiments, in which the treatment was carried out on washed bacteria.

Phenol. The mutagenic action of phenol had been discovered by Hadorn and Niggli (1946), working on Drosophila with a very special treatment technique. Other techniques had failed to give positive results. Negative results had been obtained by Dickey, Cleland, and Lotz (1949) with Neurospora. Our tests gave clear-cut positive results for this substance, and also for two closely related substances, alpha-dinitrophenol and trinitro-

TABLE 1

RESULTS OF EXPERIMENTS TESTING INDUCTION OF BACKMUTATIONS IN SH4 (STREPTOMYCIN-DEPENDENT) *E. COLI* TREATED WITH CERTAIN CHEMICALS

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Sorbic acid	0.003	3	13.	10	1.2×10^8	11.3	4	8.3×10^7	9.3
	0.002	3	12.	8	3.0×10^7	70.0	4	7.0×10^7	147.
	0.002	3	11.	8	1.1×10^8	32.7	4	8.4×10^7	35.4
	0.005	3	7.0	9	4.6×10^7	10.9	4	5.4×10^7	33.1
	0.002	3	1.9	8	2.2×10^7	54.	4	9.2×10^7	98.1
Phosphoric acid (excess)	0.008	3	29.	8	1.9×10^8	25.4	4	6.4×10^7	35.9
	0.008	3	15.	8	1.0×10^8	16.5	4	7.3×10^7	12.3
	0.010	3	1.2	8	0.3×10^7	2.2	4	8.3×10^7	21.3
Nitric acid	0.008	3	8.9	10	8.1×10^7	11.1	4	8.3×10^7	9.3
	0.005	3	8.3	8	5.3×10^7	90.2	4	7.0×10^7	147.
	0.005	3	6.9	8	6.3×10^7	16.2	4	8.4×10^7	35.4
	0.006	3	3.5	10	2.6×10^7	11.7	4	5.4×10^7	33.1
	0.005	3	2.7	9	2.2×10^7	111.1	4	9.2×10^7	98.1
Hydrochloric acid	0.0015	3	100.	5	6.0×10^7	6.7	4	4.8×10^7	10.4
	0.0015	3	33.	5	4.0×10^7	5.8	4	9.6×10^7	24.0
	0.00175	3	21.	5	2.5×10^7	6.8			24.0
	0.0075	3	11.	10	1.3×10^8	30.1			10.4
	0.00075	3	0.9	3	1.1×10^8	0.0	2	9.2×10^7	3.6
	0.0015	3	0.2	4	4.9×10^8	0.0			3.6
	0.0015	3	0.2	4	3.9×10^8	23.0	3	2.5×10^8	26.2
Boric acid	4.0	3	50.	8	1.0×10^8	27.3	6	7.0×10^7	6.0
	1.5	24	33.	8	6.0×10^8	28.9	5	1.1×10^8	7.9
	2.0	24	25.	8	4.6×10^8	28.2			7.9
	2.0	24	24.	8	3.3×10^8	82.3	6	1.0×10^8	8.9

TABLE I—*continued*

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Boric acid *(continued)*	2.0	24	17.	8	1.1×10^8	6.6	6	4.6×10^8	9.8
	2.0	24	11.	8	1.6×10^8	64.1	6	1.0×10^8	13.6
	1.5	24	11.	8	8.3×10^7	31.2	4	8.9×10^7	21.3
	1.7	24	10.	9	5.9×10^7	20.3	6	9.0×10^7	6.7
	2.0	48	9.7	8	9.2×10^7	16.4	4	7.0×10^7	5.7
	1.9	24	3.3	10	2.2×10^7	18.6			6.7
	1.8	24	2.8	8	2.1×10^7	56.6	4	7.5×10^7	30.6
Sodium hydroxide	0.013	3	86.	10	2.6×10^9	11.7	5	3.0×10^6	18.9
	0.015	3	37.	10	3.0×10^8	32.4	4	6.4×10^7	35.9
	0.02	3	27.	7	5.4×10^8	13.8	5	1.4×10^8	16.8
	0.011	3	24.	10	3.4×10^8	3.3	6	1.9×10^8	3.6
	0.0145	3	20.	10	1.9×10^8	20.2	4	8.5×10^7	8.3
	0.015	3	16.	10	1.2×10^8	16.3	4	7.3×10^7	12.3
	0.0125	3	12.	8	1.2×10^8	28.2	4	1.0×10^8	38.0
	0.0125	3	11.	8	1.0×10^8	130.7	4	9.2×10^7	98.1
	0.014	3	5.7	8	5.3×10^7	43.0	4	6.8×10^7	43.8
	0.022	3	1.3	8	2.5×10^7	21.6	5	1.1×10^8	12.1
Potassium hydroxide	0.00945	3	44.	10	2.8×10^8	10.3	6	9.0×10^7	6.7
	0.015	3	42.	8	3.8×10^8	55.5	4	9.2×10^7	98.1
	0.016	3	32.	8	3.0×10^8	43.0	4	6.8×10^7	43.8
	0.015	3	29.	8	2.0×10^8	79.2	4	7.0×10^7	147.
	0.019	3	20.	10	3.6×10^8	12.5	6	1.8×10^8	5.5
	0.018	3	18.	10	1.4×10^8	9.9	6	1.1×10^8	9.7
	0.018	3	19.	10	3.4×10^8	20.1			5.5
	0.0185	3	13.	10	1.9×10^8	6.4	6	1.9×10^8	3.6
	0.0165	3	13.	10	1.8×10^8	4.4			3.6
	0.017	3	10.	10	7.9×10^7	12.7			9.7

TABLE I (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^9 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^9 bacteria
Potassium hydroxide (*continued*)	0.019	24	8.5	10	1.5×10^9	17.8			5.5
	0.018	24	8.3	10	1.5×10^9	9.4			5.5
	0.0175	3	2.9	8	2.9×10^7	37.6	4	1.0×10^9	38.0
Ammonia	0.025	3	66.	6	2.4×10^9	4.9	2	1.8×10^9	6.8
	0.100	3	26.	9	1.1×10^9	2.6	5	2.5×10^9	3.7
	0.250	3	13.	9	3.9×10^9	10.3	5	1.7×10^9	10.0
	0.250	3	12.	8	3.1×10^8	1.6	4	1.4×10^9	2.8
	0.300	3	9.6	5	2.4×10^8	3.4	5	2.5×10^9	2.6
	0.400	3	2.3	10	1.1×10^8	8.0			2.6
	0.250	3	1.2	8	2.4×10^7	37.5	5	1.3×10^9	12.9
	0.500	3	1.0	4	1.2×10^7	41.6	5	1.5×10^9	13.3
	0.500	3	0.4	10	2.0×10^7	121.			2.6
	0.500	3	0.2	10	5.6×10^6	90.			12.9
Hydrogen peroxide	0.150	4	78.	9	1.9×10^9	19.9	8	2.2×10^9	5.0
	0.195	4	59.	8	1.3×10^9	28.7			5.0
	0.195	½	45.	6	5.2×10^8	124.	8	1.5×10^9	6.9
	0.225	½	32.	8	4.9×10^8	121.			6.9
	0.195	½	3.1	8	5.4×10^7	175.	6	1.3×10^9	4.7
	0.240	4	1.4	9	3.5×10^7	120.			5.0
	0.195	½	0.6	8	1.1×10^7	26.7			4.7
Mercuric chloride	0.00005	6	61.	9	5.8×10^8	1.7	5	1.5×10^9	0.7
	0.00030	3	40.	10	4.1×10^8	4.2	4	4.1×10^8	12.3
	0.00025	3	21.	4	3.1×10^8	5.9	4	1.5×10^8	2.0
	0.00030	3	18.	3	2.5×10^8	2.9	2	9.2×10^7	3.3
	0.00005	9	11.	9	1.1×10^8	3.7			0.7
Silver nitrate	0.000005	24	84.	9	3.3×10^9	4.8	6	2.6×10^9	2.3
	0.000100	3	79.	10	1.6×10^9	2.5	5	1.0×10^9	8.0
	0.000050	3	75.	9	1.1×10^9	6.7	7	1.1×10^9	8.6

TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Silver nitrate (*continued*)	0.000025	3	59.	9	2.3×10^8	2.1			2.3
	0.000100	3	26.	9	3.8×10^8	7.4			8.6
	0.000100	24	4.3	9	6.1×10^7	8.2			8.6
Copper sulfate	0.00020	3	88.	9	1.4×10^9	5.7	6	1.0×10^9	5.1
	0.00040	3	34.	9	4.7×10^8	3.6	8	1.2×10^9	8.8
	0.00050	3	22.	10	5.0×10^8	4.4	5	1.1×10^9	8.9
	0.00050	3	14.	9	3.8×10^8	10.6	5	1.5×10^9	2.1
	0.00050	3	12.	3	1.1×10^8	20.7	4	1.2×10^8	20.8
	0.00075	3	2.4	9	3.7×10^7	40.5			5.1
	0.00100	3	0.36	9	7.2×10^6	55.5			8.9
	0.00125	3	0.22	10	4.9×10^6	0.0			8.9
Sodium silicate	0.025	3	66.	10	1.7×10^8	5.9	5	1.3×10^8	6.3
	0.100	3	33.	5	4.2×10^8	2.4	5	1.3×10^9	5.3
	0.050	3	27.	10	6.9×10^7	8.7			6.3
	0.100	3	16.	7	3.8×10^8	6.6	5	1.7×10^8	6.1
	0.100	3	4.6	9	7.9×10^7	11.4	7	1.3×10^9	6.2
	0.150	3	3.0	9	5.1×10^7	2.0			6.2
	0.300	3	0.11	5	1.3×10^6	0.0	5	1.2×10^9	7.0
Acetic acid	0.03	3	79.	10	1.3×10^9	8.3	5	1.6×10^8	10.0
	0.03	3	36.	8	9.9×10^8	36.4	5	1.7×10^8	9.2
	0.03	3	11.	10	8.5×10^7	15.3			10.0
	0.01	24	7.2	8	7.6×10^7	19.8	6	7.9×10^7	10.1
	0.03	3	6.0	10	9.5×10^7	25.2			10.0
	0.03	3½	0.9	8	8.8×10^6	56.8	6	7.6×10^7	6.6
Lactic acid	0.010	3	40.	10	6.4×10^8	12.8	5	1.6×10^8	10.0
	0.010	3	36.	10	2.9×10^8	10.7			10.0
	0.019	3	14.	8	2.0×10^8	16.7	5	8.9×10^7	4.5

TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Lactic acid (*continued*)	0.021	3	7.5	8	1.1×10^9	40.2			4.5
	0.020	3	6.8	8	1.7×10^9	17.0	5	1.6×10^8	15.3
	0.020	3	4.6	8	6.7×10^8	32.9	5	9.2×10^7	27.3
	0.020	4	2.5	8	2.9×10^7	34.7	6	8.5×10^7	9.4
	0.020	3	1.9	8	2.6×10^7	11.4	6	1.0×10^8	4.8
Sodium lactate	2.5	3	85.	8	1.5×10^9	7.3	5	1.1×10^9	12.1
	3.0	3	61.	8	1.7×10^9	16.4	5	1.6×10^8	15.3
	2.0	24	8.3	8	8.7×10^7	5.8	6	7.9×10^7	10.1
	2.5	24	1.4	8	3.8×10^7	5.2	5	1.7×10^8	9.2
Formic acid	0.0070	3	100.	8	1.5×10^9	18.1	6	1.1×10^8	2.7
	0.0060	3	93.	8	1.4×10^9	18.4			2.7
	0.0050	3	77.	8	1.3×10^9	20.2	5	1.1×10^8	12.1
	0.0065	3	30.	8	8.2×10^8	44.0	5	1.7×10^8	9.2
	0.0060	3	20.	8	5.0×10^8	43.3	5	1.6×10^8	15.3
	0.0065	3	19.	8	4.6×10^8	24.4	5	1.5×10^8	3.3
	0.0060	3	15.	8	1.4×10^8	27.7	6	7.1×10^7	2.8
	0.0075	3	3.4	8	8.2×10^7	38.0			3.3
	0.0070	3	2.8	8	2.6×10^7	33.5			2.8
Formaldehyde	0.01	3	61.	2	7.6×10^7	400.	2	1.3×10^8	26.2
	0.01	3	58.	5	6.8×10^8	133.	4	9.4×10^8	15.5
	0.02	3	58.	5	6.8×10^8	157.			15.5
	0.01	3	48.	4	3.7×10^7	245.	4	7.6×10^7	79.
	0.01	3	48.	4	3.7×10^6	470.	4	7.6×10^6	63.
	0.01	3	21.	8	1.3×10^8	120.	4	7.3×10^7	12.3
	0.01	3	16.	8	1.0×10^8	129.			12.3
	0.02	3	9.0	7	1.5×10^8	326.			15.5
	0.04	3	1.7	10	3.2×10^8	560.			79.

TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Phenol	0.10	3	90.	10	4.4×10^8	84.5	4	2.0×10^8	74.5
	0.10	3	69.	9	1.2×10^8	2.5	5	9.5×10^8	0.6
	0.08	3	48.	8	7.4×10^8	3.4			0.6
	0.20	3	43.	9	7.5×10^8	2.5			0.6
	0.10	12	1.7	10	7.3×10^8	124.	5	2.2×10^8	74.8
	0.10	24	1.1	10	3.1×10^8	192.	4	1.1×10^8	59.5
	0.20	3	0.5	9	2.3×10^8	820.			74.5
Alpha-dinitro-phenol	0.005	3	87.	3	9.6×10^8	2.7	2	7.4×10^7	4.1
	0.020	3	72.	3	1.1×10^8	8.1	5	2.5×10^8	5.5
	0.050	3	58.	10	1.7×10^8	33.5	5	1.5×10^8	5.4
	0.020	3	46.	10	9.5×10^7	51.5	4	8.4×10^7	8.5
	0.005	3	37.	10	2.3×10^8	25.1	5	3.1×10^8	12.5
	0.050	3	32.	10	6.6×10^7	56.0			8.5
	0.025	3	31.	9	1.7×10^8	14.2			12.5
	0.040	3	23.	10	4.8×10^7	25.0			8.5
	0.050	3	18.	10	9.1×10^7	37.4			5.5
	0.100	3	3.0	9	8.2×10^7	44.0	5	1.5×10^8	7.2
	0.100	3	2.6	10	7.3×10^7	97.0	5	1.4×10^8	7.3
	0.100	3	2.5	9	1.2×10^8	6.0			5.5
	0.100	3	2.4	7	3.5×10^7	65.5	4	8.4×10^8	7.6
	0.100	3	1.7	8	4.8×10^7	79.0	4	1.4×10^8	2.8
Trinitro-phenol	0.010	3	94.	8	1.9×10^8	14.9	5	1.3×10^8	14.3
	0.018	3	34.	8	4.9×10^8	97.3	6	1.1×10^8	9.3
	0.017	3	7.4	8	5.8×10^7	132.	6	5.8×10^7	3.4
	0.017	3	4.2	8	6.2×10^7	27.4	5	8.9×10^7	4.5
	0.018	3	1.9	8	1.9×10^7	68.4	6	7.8×10^7	7.7

TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Sodium salicylate	2.0	3	62.	5	4.2×10^7	16.7	5	7.0×10^7	20.3
	1.0	3	38.	3	9.5×10^7	5.3	5	4.0×10^8	5.8
	2.0	3	10.	8	3.2×10^8	0.0	5	2.0×10^8	0.7
	3.0	3	0.03	10	2.2×10^8	0.0	5	4.0×10^8	5.8
Carbamate (methyl)	4.0	48	100.	7	3.0×10^8	8.0	5	1.7×10^7	6.1
	8.0	3	69.	8	1.4×10^8	14.4	6	7.8×10^7	7.7
	7.0	25	10.	8	1.8×10^8	5.5	6	1.3×10^8	10.6
	8.0	24	5.5	8	1.1×10^8	9.1	6	8.6×10^7	19.9
	8.0	24	4.6	8	8.6×10^7	4.7	6	7.0×10^7	5.7
	7.0	29	2.2	5	2.4×10^7	12.6			10.6
Carbamate (ethyl) =Urethane	5.0	3	62.	10	1.4×10^8	18.6	5	1.1×10^8	7.9
	6.0	3	56.	8	1.3×10^8	26.5	5	1.4×10^8	16.8
	4.0	3	27.	10	4.8×10^8	8.4	6	1.8×10^8	5.5
	3.5	3	25.	10	4.5×10^8	6.6			5.5
	7.0	3	10.	8	1.7×10^8	41.4	5	1.1×10^8	12.1
	7.0	3	8.3	8	1.7×10^8	53.3	5	1.3×10^8	14.3
	7.0	4	8.2	8	2.3×10^8	22.5	5	1.7×10^8	9.2
	7.0	4	4.7	7	5.6×10^7	98.5	5	8.5×10^7	8.3
	7.0	4½	2.7	8	2.0×10^7	40.0	6	5.8×10^7	3.4
Carbamate (*n*-propyl)	2.15	3	52.	7	4.6×10^8	28.0	6	7.6×10^7	6.6
	2.00	3	50.	8	7.0×10^8	11.9	6	1.1×10^8	7.5
	2.00	3	35.	8	3.6×10^8	29.9	6	7.8×10^7	6.4
	2.00	3½	32.	8	6.6×10^8	8.3	6	1.6×10^8	3.9
	2.00	3½	30.	8	7.2×10^8	10.6	6	1.8×10^8	2.2
	2.10	3½	29.	6	5.2×10^8	16.4			2.2
	2.10	3	24.	8	2.7×10^8	23.0	6	8.5×10^7	9.4
	2.10	3½	4.1	8	9.8×10^7	17.4	5	1.5×10^8	3.3

TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Carbamate (*n*-propyl) (*continued*)	2.10	3½	3.9	8	8.1×10^7	9.9			3.9
	2.10	3	2.9	8	7.0×10^7	24.2			3.3
	2.10	3½	2.5	8	3.2×10^7	34.8	6	9.7×10^8	12.3
	2.10	4	1.4	8	3.4×10^7	23.8			3.5
Carbamate (*n*-butyl)	0.50	3	85.	8	8.6×10^8	21.4	6	7.8×10^7	7.7
	0.76	3½	44.	7	9.2×10^8	5.3	6	1.8×10^8	2.2
	0.75	3½	29.	8	7.0×10^8	5.6	5	1.5×10^8	3.3
	0.75	3½	20.	8	4.2×10^8	8.3	6	1.6×10^8	3.9
	0.75	3½	19.	7	1.7×10^8	20.9	6	7.6×10^7	6.6
	0.75	3½	17.	8	2.6×10^8	7.4	6	1.1×10^8	2.7
	0.75	3	16.	8	1.6×10^8	10.0	7	7.6×10^7	9.2
	0.80	3½	4.9	8	1.2×10^8	5.9			3.3
	0.75	3½	3.4	8	4.5×10^7	8.9	6	9.7×10^7	12.3
	0.75	3½	1.4	8	1.5×10^7	0.0	6	7.8×10^7	6.4
Carbamate (isoamyl)	0.25	3	100.	8	2.0×10^9	8.4	6	1.6×10^8	3.9
	0.30	3	100.	8	1.6×10^9	11.3	6	1.1×10^8	2.7
	0.35	3	57.	8	8.5×10^8	8.0			2.7
	0.35	3	54.	8	1.1×10^9	9.4			3.9
	0.36	3	26.	8	6.2×10^8	6.3	6	1.8×10^8	2.2
	0.38	3	6.3	8	1.5×10^8	7.2			2.2
	0.37	3	5.8	8	1.4×10^8	7.0			2.2
Neutral red (in light)	0.00025	3	50.	9	7.6×10^8	57.3	9	1.5×10^9	6.6
	0.00100	3	43.	9	6.6×10^8	118.			6.6
	0.00050	3	24.	8	3.2×10^8	122.			6.6
	0.00050	12	0.2	8	1.2×10^6	178.	8	6.7×10^8	10.8
Neutral red (in the dark)	0.00050	24	90.	9	4.2×10^8	17.2	6	4.6×10^8	10.1
	0.00050	12	86.	8	9.6×10^7	10.6	7	9.8×10^7	9.8

TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Neutral red (in the dark) (*continued*)	0.00050	24	83.	12	6.5×10^7	15.4	10	6.5×10^7	15.4
	0.00100	24	71.	10	4.6×10^7	17.4			15.4
	0.00050	12	70.	15	9.0×10^8	10.8	8		10.8
Acriflavine	0.0015	3	39.	10	4.8×10^8	78.3	4	1.0×10^8	14.4
	0.0030	3	28.	10	3.4×10^8	59.1			14.4
Caffeine alkaloid	2.10	3	72.	7	1.3×10^8	15.5	6	7.6×10^7	6.6
	0.60	48	44.	7	7.8×10^8	31.1	6	1.5×10^8	9.3
	2.10	24	34.	8	1.7×10^8	30.3	6	3.7×10^7	10.9
	0.90	48	15.	8	3.9×10^8	21.8	5	1.1×10^8	7.9
	1.00	48	15.	8	1.3×10^8	32.1	6	6.7×10^7	17.9
	0.50	48	12.	10	7.0×10^7	13.0	6	7.1×10^7	5.6
	0.60	48	5.1	8	9.3×10^7	34.6			7.9
	1.09	48	3.8	8	2.0×10^7	20.4	6	3.9×10^7	2.5
Necrosin	0.20	3	100.	8	4.6×10^8	23.6	6	1.4×10^7	6.9
	0.05	3	100.	8	4.2×10^8	26.0			6.9
	0.40	3	100.	8	1.2×10^8	16.4	6	1.0×10^8	9.9
	1.30	3	44.	8	8.5×10^8	17.8	6	1.4×10^8	8.4
	0.65	3½	3.4	7	3.9×10^7	172.	6	9.8×10^7	4.1

phenol (picric acid). The mutagenicity of phenol, however, was evident only after treatments that left very few survivors.

Sodium salicylate. Not mutagenic.

Carbamates. It was known from Oehlkers' work (1943) that some carbamates were able to induce chromosomal aberrations. Vogt (1948) had discovered that ethyl carbamate (urethane) showed mutagenic action in Drosophila. Since then, carbamates had been used by Bryson (unpublished) to induce mutations to phage resistance in *E. coli.* In our experiments, methyl carbamate gave negative results; ethyl and *n*-propyl carbamate gave good positive results; and *n*-butyl carbamate and isoamyl carbamate showed much weaker mutagenic effect.

Neutral red. Luther (1939) had found that neutral red was able to produce chromosomal aberrations. Buzzati (1947), treating Drosophila eggs with this substance, had observed an increase in frequency of mosaic flies. In our tests it seemed at first impossible to obtain low survival ranges, because treatment with concentrations higher than 0.001 per cent produced heavy clumping, and increases in length of treatment did not appreciably affect the number of survivors. Therefore no clear-cut positive results were obtained with the standard procedure. Since neutral red is a photodynamic substance, however, we then tried exposing the bacteria to light during treatment (a 60-watt bulb at a distance of about 10 cm), and finally got the desired survival ranges and with them a very large increase in frequency of mutants. Light had no effect on the controls. Similar results were reported by Kaplan (1948, 1949, 1950a, 1950b), treating *Bacterium prodigiosum* and *E. coli* with erythrosine.

Acriflavine. This substance was known to be a mutagenic agent for both bacteria and Drosophila (Witkin, 1947). Our tests also showed clear-cut positive results.

Caffeine. Caffeine had been found to be mutagenic for Ophiostoma (biochemical mutations) by Fries and Kihlman (1948) and for *E. coli* (mutations to phage resistance) by Witkin (Demerec, Wallace, and Witkin, 1948). Our experiments confirmed these results.

Necrosin. Necrosin is the name given by Menkin (1943) to the potent injurious euglobulin fraction of the exudate of inflamed tissues of vertebrates. A similar substance has subsequently been found by him in severely damaged tissues of some invertebrates (Menkin, 1949). The sample of necrosin used in our experiments was received from Dr. Menkin. It was found to be mutagenic, particularly in experiments where the proportion of survivors was low.

DISCUSSION

Streptomycin-dependent (Sd-4) bacteria, when plated on broth-agar medium without any streptomycin, undergo several divisions (residual growth) and then stop growing. The concentration of streptomycin in the medium in which the parent bacteria are grown is the most significant of the known factors influencing the number of divisions. For example, bacteria grown

in broth medium containing 10 micrograms of streptomycin per milliliter, when plated on medium without streptomycin, will pass through 2-3 divisions; whereas bacteria grown in medium containing 25 micrograms of streptomycin will pass through 3-4 divisions. It has been found that very few, if any, nondependent back-mutants (background mutants) are present in cultures grown in broth with streptomycin, and therefore almost all such back-mutants appearing on the streptomycin-free plates can be assumed to originate during the residual growth. The almost complete absence of background mutants reduces the possibility that differential killing may be responsible for the increased proportion of back-mutants found among the survivors of treatment with certain chemicals; and this, together with the facts that residual growth can be dependably regulated in untreated material and that it allows expression of the back-mutations induced by treatment, makes the Sd-4 method especially suitable for studies of induced mutability.

Since spontaneous back-mutations occur during residual growth, any factor that increases this growth should increase the number of mutants. Therefore, in experiments such as these described here, it is necessary to be sure that any observed increase in mutant frequency is an effect of the treatment on the treated bacteria and not an effect of the treatment on the extent of residual growth. It is not likely that the chemicals used in these experiments would increase the residual growth. In the first place, they were usually applied in nonphysiological, toxic doses: Furthermore, the streptomycin requirement of the dependent bacteria is highly specific (Demerec, 1950), and ability to substitute for it would be quite unexpected in an unrelated chemical. However, even supposing that this had occurred, it would have been detected, either by the appearance of heavy residual growth on the plates of treated bacteria or through the microscopic check of the extent of residual growth that was made in all doubtful cases.

The analysis of data on the mutation-producing chemicals, presented in table 2, indicates that there were considerable differences of behavior among them. Some (ammonia, copper sulfate, and necrosin) were mutagenic only in treatments that left less than 5 per cent survivors; whereas others (hydrogen peroxide, formaldehyde, alpha-dinitrophenol, and neutral red in light) showed effects in treatments that allowed more than 50 per cent survival. Formaldehyde appears to be the most potent mutagen on the list. A treatment that left 61 per cent survivors resulted in 400 mutants per 10^8 treated bacteria; and two other treatments, each leaving 58 per cent survivors, gave 133 and 157 mutants, whereas in the controls there were only 25.2 and 15.5 mutants per 10^8.

Of the 31 chemicals listed in table 1, 19 were found to be mutagenic (table 2), and the following 12 were not: sulfuric, phosphoric, nitric, and hydrochloric acids; sodium and potassium hydroxides; mercuric chloride; silver nitrate; sodium silicate; sodium lactate; sodium salicylate; and methyl carbamate. Since the potency of the different mutagenic chemicals ranges from slight to strong, and, at least in the case of one mutagen that has been studied intensively (manganous chloride), can be shifted from one

TABLE 2

SUMMARY OF THE DATA ON MUTAGENIC CHEMICALS, SHOWING HIGHEST AND LOWEST FREQUENCIES OF MUTANTS OBSERVED IN EXPERIMENTS USING DIFFERENT TREATMENTS (EXPRESSED IN PERCENTAGES OF SURVIVORS)

	Survivors (per cent)			Controls
	50–100	5–50	less than 5	
	No. of mutants per 10^8 bacteria			
Boric acid	27	16–82	19–57	6–31
Ammonia	5	2–10	38–121	3–13
Hydrogen peroxide	20–29	121–124	27–175	5–7
Copper sulfate	6	4–21	40–56	2–21
Acetic acid	8	15–36	57	7–10
Lactic acid	...	11–40	11–35	5–27
Formic acid	18–20	24–44	38–39	3–15
Formaldehyde	133–400	120–326	560	12–79
Phenol	3–85	...	124–820	1–75
Alpha-dinitrophenol	3–34	14–56	6–97	3–13
Trinitrophenol	15	9–132	27–68	3–14
Carbamate, ethyl	19–27	7–53	40–99	6–20
n-propyl	12–28	8–30	10–35	2–12
n-butyl	21	5–21	6–9	2–12
isoamyl	8–11	6	7	2–4
Neutral red, light	57	118–122	178	7–11
dark	11–17	...	...	7–15
Acriflavine	...	59–78	...	14
Caffeine	16	13–35	20	3–18
Necrosin	16–26	18	172	4–10

extreme to the other by simple pretreatments of the bacteria, it seems very likely that the chemicals listed above as nonmutagens might become mutagenic if the treatment were made in a certain way. At any rate, examination of the list of mutagens in the table shows that mutagenicity does not belong to any one group of chemicals but is distributed widely among the different groups tested. Chemicals with such different properties as boric acid, ammonia, hydrogen peroxide, copper sulfate, acetic acid, formaldehyde, and phenol are on the list of mutagens. This fact, together with the indicated possibility that chemicals now considered noneffective would become mutagenic if appropriate conditions of treatment were found, leads us to conclude that genetic changes may be induced by most agents that are able to enter a living cell, upsetting some of its metabolic functions. It seems very likely that the genetic effect of a chemical is not direct but induced through changes in the cytoplasm, which in turn affect the genes. If this is so, it should be very difficult to establish what is the final reaction producing a change in a gene. It may well be that similar final reactions may be initiated by very different primary changes.

In this study we were considering back-mutations from Sd-4; and there is evidence to indicate that a majority of these may occur at one gene locus (Demerec, 1950; Newcombe and Nyholm, 1950). Several experiments by

others, however, based on observation of mutations to T1 phage resistance, have tested the mutagenicity of a few of the chemicals we used. Witkin (Witkin, 1947; Demerec, Wallace, and Witkin, 1948) found that acriflavine and caffeine were mutagenic; and Bryson (in manuscript), in extensive experiments with several carbamate compounds, showed that ethyl carbamate (urethane) is mutagenic. It therefore appears very probable that the mutagenic effect observed by us was not specific, but that treatment with these chemicals increases the mutation rate of the whole genome.

It should be kept in mind that we are dealing here with genetic changes that occur spontaneously with a rate of the order of one per 10^8 cell generations, a considerably lower rate than could be detected directly in germ cells of multicellular organisms. Since each cell of a multicellular organism is a genetic entity, however, in which mutations may occur spontaneously or be induced by various agents, the likelihood of mutation in some of the somatic cells of a higher organism is good, if a large number of these cells are exposed to the action of a mutagenic chemical. This likelihood is further increased by the fact that each cell contains several thousand genes, in any one of which a mutation may be induced.

SUMMARY

Thirty-one chemicals, representing various organic and inorganic groups, were tested for ability to induce back-mutations from streptomycin dependence (Sd-4) to nondependence in *E. coli*. Nineteen were found to be mutagens. It was demonstrated that mutagenicity is not a specific property of any one group of chemicals, but appears among widely different groups. Chemicals having such different properties as boric acid, ammonia, hydrogen peroxide, copper sulfate, acetic acid, formaldehyde, and phenol were found to be mutagenic, indicating that genetic changes may be induced by many agents that are able to enter the living cell and upset its metabolic functions.

LITERATURE CITED

Auerbach, C., 1949, The mutagenic mode of action of formalin. Science 110: 419–420.

Bertani, G., 1951, A method for detection of mutations, using streptomycin dependence in *Escherichia coli*. Genetics 36, in press.

Buzzati-Traverso, A., 1947, Sull'effetto mutageno del fosfato di sodio. Ric. Sci. Ricer. 17: 1383–1384.

Demerec, M., 1950, Reaction of populations of unicellular organisms to extreme changes in environment. Amer. Nat. 84: 5–16.

1951, Studies of the streptomycin-resistance system of mutations in *E. coli*. Genetics 36, in press.

Demerec, M., B. Wallace, and E. M. Witkin, 1948, The gene. Carnegie Inst. Wash. Yr. Bk. 47: 169–176.

Dickey, F. H., G. H. Cleland, and C. Lotz, 1949, The rôle of organic peroxides in the induction of mutations. Proc. nat. Acad. Sci. 35: 581–586.

Fries, N., and B. Kihlman, 1948, Fungal mutations obtained with methyl xanthines. Nature 162: 573–574.

Goldat, S. J., and V. N. Beliaieva, 1935, Artificial induction of mutations in *Drosophila melanogaster* through hydrochloric acid. (Russian) Biol. Zh. (Moscow) 4: 379–384.

Hadorn, E., and H. Niggli, 1946, Mutations in Drosophila after chemical treatment of gonads in vitro. Nature 157: 162–163.

Kaplan, R. W., 1948, Auslösung von Mutationen durch sichtbares Licht mit vitalgefärbten *Bacterium prodigiosum*. Naturwiss. 35: 127.

1949, Mutations by photodynamic action in *Bacterium prodigiosum*. Nature 163: 573–574.

1950a, Auslösung von Phagenresistenzmutationen bei *Bacterium coli* durch Erythrosin mit und ohne Belichtung. Naturwiss. 37: 308.

1950b, Mutationsauslösung bei *Bacterium prodigiosum* durch sichtbares Licht nach Vitalfärbung mit Erythrosin. Arch. Mikrobiol. 15: 152–175.

Kaplan, W. D., 1948, Formaldehyde as a mutagen in Drosophila. Science 108: 43.

Law, L. W., 1938, The effects of chemicals on the lethal mutation rate in *Drosophila melanogaster*. Proc. nat. Acad. Sci. 24: 546–550.

Lobashov, M. E., 1937, Über die Natur der Einwirkung der chemischen Agentien auf der Mutationsprozess bei *Drosophila melanogaster*. Genetica 19: 200–241.

Lobashov, M. E., and F. A. Smirnov, 1934, Über die Wirkung der Essigsäure auf Non-disjunction und Transgenationen bei *Drosophila melanogaster*. Dokl. Acad. Sci. URSS, N. S. 2: 307–311. (Russian with German summary.)

Luther, W., 1939, Über eine der Röntgenwirkung ähnliche Chromosomenschädigung in den Epidermis-Zellen von Amphibienlarven nach Behandlung mit Neutralrot. Klin. Wochschr. 1: 682–683.

Magrzhikovskaja, K. V., 1936, The influence of $CuSO^4$ on the mutation process in *Drosophila melanogaster*. Bull. Biol. Med. Exp. U.R.S.S. 2: 90–92.

Menkin, V., 1943, Chemical basis of injury in inflammation. Arch. Path. 36: 269–288.

1949, Cellular injury in some invertebrates. Physiol. Zool. 22: 124–131.

Newcombe, H. B., and M. H. Nyholm, 1950, The inheritance of streptomycin resistance and dependence in crosses of *Escherichia coli*. Genetics 35: 603–611.

Oehlkers, F., 1943, Die Auslösung von Chromosomenmutationen in der Meiosis durch Einwirkung von Chemikalien. Z. ind. abst. Ver. 81: 313–341.

Rapoport, J. A., 1946, Carbonyl compounds and the chemical mechanism of mutations. Dokl. Acad. Sci. URSS, N.S. 54: 65–67.

Stone, W. S., O. Wyss, and F. Haas, 1947, The production of mutations in Staphylococcus by irradiation of the substrate. Proc. nat. Acad. Sci. 33: 59–66.

Vogt, M., 1948, Mutationsauslösung bei Drosophila durch Äthylurethan. Experientia 4: 68–69.

Witkin, E. M., 1947, Mutations in *Escherichia coli* induced by chemical agents. Cold Spring Harbor Symp. Quant. Biol. 12: 256–269.

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RAPID UPTAKE OF 1-^{14}C ACETATE BY THE ADULT RAT BRAIN 15 SECONDS AFTER CAROTID INJECTION

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SUMMARY

1-^{14}C Acetate was injected into the carotid artery of adult rats followed by decapitation after 15 sec. Brain, liver and blood lipids were extracted. Analysis of the lipids showed the following:

1. There was considerably more uptake of radioactivity in the brain as compared to the liver or the plasma.

2. High radioactivity in the cholesterol fraction indicating rapid synthesis from radioactive acetate.

3. Incorporation of radioactivity into all major polar lipid fractions of the brain lipids including sphingomyelin and cerebrosides, considered to be myelin lipids. Phosphatidyl serine was the most highly labeled component.

4. Palmitic acid isolated from the brain was synthesized *de novo* from acetate and stearic acid was formed by chain elongation.

All these metabolic reactions occurring so rapidly in the brain are discussed in view of the older concept that adult brain is a tissue characterized by slow metabolism.

INTRODUCTION

Despite work throughout the last decade, in which numerous workers have shown appreciable amounts of incorporation of radioactive tracers into the brain, the general impression that the lipid metabolism of the brain is rather slow persists. Earlier literature[1] implied that ^{32}P injected intravenously or intraperitoneally was incorporated very slowly into brain lipids mainly because the blood brain barrier restricted the entry of the phosphate ion. However, once it permeated into the brain tissue there was rapid incorporation into phospholipids within 30 min. When heavy water[2], ^{3}H-labeled fatty acids[3,4], and cholesterol[5] were administered to adult rats, very

List of Abbreviations used: PC = Phosphatidylcholine; PE = Phosphatidylethanolamine; PS = Phosphatidylserine; Cereb. = Cerebroside; Cera. = Ceramid; Cereb. Sulf. = Cerebroside sulfate;

little, if any, label was incorporated into brain total lipids, although very young rats took up the label rapidly. Discussing the biosynthesis and turnover rates, DAVISON[6] concluded that although the CNS contains large amounts of lipids, biosynthesis and mean turnover rates of typical myelin lipids including cholesterol are quite slow. Against this background a very recent communication from OLDENDORF[7] shows a rapid uptake of many essential amino acids only 15 sec after a carotid artery injection of the test compound. The present work was designed to examine in a similar way the rapid uptake and incorporation of [1-^{14}C]acetate, given *via* a carotid artery injection, by the adult rat brain.

MATERIALS AND METHODS

Animals

Six adult albino Wistar rats weighing approximately 400 g were used in the study; they had free access to food and water.

Tracer

10 μC of [1-^{14}C]acetate (spec. act. 45 mC/mM, New England Nuclear Corp., Des Plaines, Ill.) dissolved in 0.2 ml of water was injected into the carotid artery of the adult rats as one rapid bolus with decapitation 15 sec later as described by OLDENDORF[8]. Brain tissue was quickly dissected out, placed in a 3-ml syringe and extruded into a previously weighed vial containing 5 ml of chloroform–methanol (2:1, v/v). Similarly, a portion of the liver from each lobe was quickly added to another vial containing the same solvent. The total time between injection and extrusion of the tissue into chloroform–methanol was never more than 45–50 sec. Blood was collected into heparinized tubes and centrifuged immediately for 3 min. Plasma was separated and frozen in dry ice immediately until analyzed.

Extraction of the lipids from brain, liver and plasma was done by the method of FOLCH *et al.*[9]. The total lipids were extracted from the tissue of the six rats separately and mean values with standard deviations are given in Table I. The fractionation of the lipids was done on pooled samples. Fractionation of total lipids, isolation of pure palmitic and stearic acid, decarboxylation of the pure fatty acids[10] *etc.* has been described in earlier communications[11,12]. A packard TriCarb liquid scintillation spectrometer Model 574, operating at 80% efficiency was used for all radioactivity determinations.

RESULTS

TABLE I

INCORPORATION OF [1-^{14}C]ACETATE BY RAT BRAIN, LIVER AND PLASMA LIPIDS 15 SEC AFTER CAROTID ARTERIAL INJECTION

	Spec. act. total lipids (counts/min per mg)	*Radioactivity of total lipids (counts/min per g fresh weight of tissue)*	*% of given dose (µC/100 g of body weight) recovered in total lipids*	*Cholesterol spec. act. (counts/min per mg)*	*Polar lipids (phospholipids sphingolipids) (counts/min per mg)*
Blood plasma	108 ± 121	467	0.008		--
Brain	320 ± 61	34828	0.64	86	204
Liver	31 ± 25	1241	0.02	105	54

Table I shows the data regarding uptake and incorporation of radioactivity from injected [1-^{14}C]acetate. The values represent the average of six determinations worked out separately. The specific activity of total lipids of the brain was much higher than those of the liver or the plasma. The total radioactivity expressed as counts/min of total lipids per g of wet brain is about 30 times higher in the brain than in the liver. The neutral lipid fraction from the brain was less than 1% of the brain total lipids but had extremely high radioactivity. This fraction was not characterized in the present study. The polar lipids of the brain, comprising phospholipids and sphingolipids, had higher radioactivity than cholesterol, whereas the opposite was true in the case of the liver.

Table II shows the radioactivity values of various polar lipid fractions isolated by column chromatographic methods. The data obtained 24 h after intraperitoneal injection of acetate are included for comparison. It may be seen that the interval between administration of dose and sacrifice of the animal has a profound influence on the pattern of labeling in so far as the specific activities of various components are concerned. The specific activity of phosphatidyl choline was highest in the 24-h experiment whereas phosphatidyl serine had maximum specific activity in the 15-sec experiment. It may be noted here that both sphingolipids, cerebrosise and sphingomyelin, had higher specific activity after 15 sec than after 24 h despite the fact that an approximately 100-fold higher dose of radioactive acetate was injected intraperitoneally in the 24-h experiment.

TABLE II

SPECIFIC RADIOACTIVITY OF POLAR LIPIDS AFTER ADMINISTRATION OF [1-^{14}C]ACETATE

	Specific activity (counts/min per mg)		
	Brain		*Liver*
	24 h	*15 sec*	*15 sec*
Phosphatidyl choline	130	86	43
Phosphatidyl ethanolamine	75	45	35
Phosphatidyl serine	46	239	5
Sphingomyelin	30	59	15
Ceramid	—	138	—
Cerebroside	51	138	—
Cerebroside sulfate	—	0	—

The specific activities of the various phospholipids obtained by fractionation of liver total lipids shows that phosphatidyl choline was the most active fraction and the pattern is somewhat similar to that of the brain seen in the 24-h experiment but very much different from that of the brain in the 15-sec experiment.

The percent distributions of radioactivity of palmitic and stearic acids isolated from the brain total fatty acids are shown in Table III. The distribution of radioactivity in palmitic acid is almost identical irrespective of the period between injection of the radioactive tracer and sacrifice of the animal. If palmitate is synthesized *de novo* from [1-^{14}C]acetate every odd carbon is labeled and the carboxyl carbon would have 1/8 or 12.5% of the total activity[12]. The % relative carboxyl activity of brain palmitic acid is very close to the calculated value of *de novo* synthesis of palmitate. Higher activity in the carboxyl carbon of stearic acid indicates chain elongation and there is a steady decrease in the % relative carboxyl activity as the interval between administration of the dose and sacrifice of the animal increases.

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN PALMITIC AND STEARIC ACID AFTER INJECTING 1-^{14}C ACETATE i.p., intraperitoneal; i.c., intracarotid; RCA, relative carboxyl activity (radioactivity in –COOH × 100/radioactivity in total fatty acids).

Dose given	*Period between injection of dose and sacrifice of rats*	*Fatty acid isolated from brain total lipids*			
		Palmitic acid		*Stearic acid*	
		Spec. act. (counts/min per mg)	*% RCA*	*Spec. act. (counts/min per mg)*	*% RCA*
[1-^{14}C] Acetate (i.p.)	24 h	—	14.1	—	20.6
[1-^{14}C] Acetate (i.p.)	4	—	13.5	—	25.6
[1-^{14}C] Acetate (i.c.)	15 sec	590	13.4	91	43.1

DISCUSSION

The earlier findings regarding relatively very slow uptake of intravenously administered ^{32}P (see ref. 1) were explained not so much as due to slow metabolism of the brain but to the slow penetration of ^{32}P across the blood brain barrier[13]. KISHIMOTO AND RADIN[11] came to the conclusion that in adult animals, the blood brain barrier causes the brain to come out second best to liver when there is a competition for intraperitoneally administered isotopic precursors. For this reason most workers prefer to use young rats, 10–15 days old. Thus, in a classical experiment to show the inertness of brain myelin cholesterol DAVISON AND WAJDA[12] injected 4-^{14}C-cholesterol into rabbits and chickens soon after birth and found the label in the same position even after as long a period as 375 and 502 days, respectively. Thus, while observing slow uptake or persistance of the label, the age of the experimental animal[11], as well as effect of the blood brain barrier on the penetration of the compound has to be considered[25]. In a series of papers from this laboratory[11,16,17] we have deliberately chosen adult animals and shown a direct uptake of long chain fatty acids, both saturated and polyunsaturated. The uptake of radioactivity in all these experiments was considerably higher in the liver lipids than in the brain, which agrees with the conclusions of KISHIMOTO AND RADIN[11]. However, we now feel that the time interval and route of administration are also very important factors. LEVIN AND KLEEMAN[18], emphasizing the routes of entry of compound into the brain, have stressed the importance of the sink action of CSF when compounds are presented to the brain by way of the blood.

The route of entry chosen in the present work does seem to have a profound effect on the uptake of radioactivity. In the present study, the tracer was injected directly into the common carotid artery in one quick pulse so that the brain would have the first chance at the metabolic turnover of the tracer. During the next 15 sec before decapitation, the blood flow was unimpeded. This would ensure flushing of any tracer still remaining in the blood capillary and not taken up by the brain. OLDENDORF[8] has calcualted that approximately 80% of the injection is distributed to the brain. Thus, out of the 150 μC of 1-^{14}C acetate that was injected, only a little over 1 μC actually was available to the brain. In spite of this small amount, the brain total lipids incorporated a fairly good amount of the label and, of more significance, the

specific activity of brain lipids was considerably higher than that of the liver total lipids. In the adult animals this is the first time that we have observed this relationship. The total ^{14}C radioactivity in the brain per g of tissue is about 28 times higher than that in the liver. Part of this could be due to differences in the blood flow (ml/min per g) to the two organs. The total radioactivity of blood lipids per ml of plasma was so low that it could not account for the total radioactivity of brain lipids per g of tissue, which rules out contamination from trapped blood.

Another significant fact was the extremely short interval of 15 sec between administration of the tracer and decapitation and 45 sec before the tissue was extruded into chloroform–methanol, thus preventing any further metabolic reactions in the dissected tissue. It was observed that within this very small interval, radioactive acetate was incorporated into brain total lipids, including cholesterol. The rapid synthesis of cholesterol, as evidenced by fairly high specific activity in the adult animal, confirms the observations of KABARA[19], who showed that there must be several compartments of sterol metabolism, one or more of which has a very rapid turnover.

The radioactivity from 1-^{14}C acetate was incorporated into all major polar lipids of the brain within 15–45 sec. Cerebroside, sphingomyelin, and cholesterol have been classified as typical myelin lipids[20,21]. Activity from 1-^{14}C acetate was found in these fractions also within the short interval. In a recent paper AGRAWAL *et al.*[22] have reported incorporation of 1-^{14}C acetate into myelin lipids within 10 min, but the animals used were only 10 days old.

The differences in the specific activities of various polar components of brain lipids in the 24-h experiment as compared to the 15-sec experiment shows that the turnover rates of these fractions must be different. This observation does not agree with that of DAVISON[6], who has proposed that all myelin sheath constituents (proteins and lipids) have the same turnover rate. However, it may be pointed out that extremely short periods between injection and sacrifice of animals are considered in the present study unlike the long periods considered in the study by DAVISON[6]. SMITH[23] has found that for periods ranging from 24 h to 56 days different myelin constituents turn over at distinctly different rates. For example she found that inositol phosphatide showed the highest degree of metabolic activity, whereas sphingomyelin, phosphatidyl ethanolamine, phosphatidyl serine and cerebroside incorporated radioactive carbon to a moderate extent. This agrees with the *in vitro* work by YAGIHARA *et al.*[24].

In the present study, phosphatidyl serine was the most radioactive component in the brain polar lipids, when the animals were decapitated within 15 sec, but not if the animals were killed after 24 h. ABDEL-LATIF AND ABOOD[25] in their studies on developing rat brain found that radioactivity from U-^{14}C serine was incorporated mostly in phosphatidyl serine. If the serine in phosphatidyl serine had been decarboxylated, it would have given rise to high activity in phosphatidyl ethanolamine. That such reactions are not major pathways in the brain was further confirmed from the work of ANSELL AND SPANNER[26]. This may explain the low activity of phosphatidyl ethanolamine in the 15-sec experiment as against high radioactivity of phosphatidyl serine fraction; but the high radioactivity observed in phosphatidyl serine fraction itself needs further work for elucidation.

More recently, ANSELL AND SPANNER[27] have referred to a significant turnover rate of the ethanolamine moiety in brain myelin.

The theoretical value of % relative carboxyl activity for the *de novo* synthesis of palmitic acid is 12.5%, which is very close to the experimental value obtained in the 15-sec experiment. Thus, one can conclude that within this short period palmitic acid was synthesized *de novo* from 1-^{14}C]acetate. Chain elongation of the palmitic acid to stearic acid by addition of an acetate unit is also seen within 15 sec, since the experimental value of 43.1% relative carboxyl activity is much higher than the theoretical value of 11.1% for the *de novo* synthesis of stearic acid.

In conclusion, the present study shows a substantial uptake of 1-^{14}C acetate injected intracarotid by the adult rat brain within a very short period of 15 sec between injection and decapitation. Further, the radioactive carbon was incorporated into major polar lipid fractions including cholesterol, cerebroside and sphingomyelin considered as typical myelin lipids.

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REFERENCES

1 R. M. C. Dawson and D. Richter, *Proc. R. Soc. London Ser. B.*, 137 (1950) 252.
2 H. Waelsch, W. M. Sperry and V. A. Stoyanoff, *J. Biol. Chem.*, 135 (1940) 291.
3 B. Cavanaugh and H. S. Raper, *Biochem. J.*, 33 (1939) 17.
4 W. H. Sperry, H. Waelsch and V. A. Stoyanoff, *J. Biol. Chem.*, 135 (1940) 281.
5 K. Bloch, B. H. Berg and D. Rittenberg, *J. Biol. Chem.*, 149 (1943) 511.
6 A. N. Davison, in R. Paoletti and D. Kritchevsky, *Advances in Lipid Research*, Vol. 3, Academic Press, New York, 1965, p. 171.
7 W. H. Oldendorf, *Proc. Soc. Exp. Biol. Med.*, 139 (1971) 385.
8 W. H. Oldendorf, *Brain Res.*, 24 (1970) 372.
9 J. Folch, M. Lees and G. H. Sloane-Stanley, *J. Biol. Chem.*, 226 (1957) 497.
10 R. O. Brady, R. M. Bradley and E. G. Trams, *J. Biol. Chem.*, 235 (1960) 3093.
11 G. A. Dhopeshwarkar, R. Maier and J. F. Mead, *Biochim. Biophys. Acta*, 187 (1969) 6.
12 G. A. Dhopeshwarkar and J. F. Mead, *Biochim. Biophys. Acta*, 187 (1969) 401.
13 G. E. Vladimirov, in H. Waelsch, *Biochemistry of the Developing Nervous System*, Academic Press, New York, 1955, p. 218.
14 Y. Kishimoto and N. S. Radin, *Lipids*, 1 (1966) 47.
15 A. N. Davison and M. Wajda, *Nature*, 185 (1959) 1666.
16 G. A. Dhopeshwarkar and J. F. Mead, *Biochim. Biophys. Acta*, 210 (1970) 250.
17 G. A. Dhopeshwarkar, C. Subramanian and J. F. Mead, *Biochim. Biophys. Acta*, 231 (1971) 18.
18 E. Levin and C. R. Kleeman, *Proc. Soc. Exp. Biol. Med.*, 135 (1970) 685.
19 J. J. Kabara, *J. Am. Oil Chem. Soc.*, 42 (1965) 1093.
20 A. C. Johnson, A. R. McNabb and R. J. Rossiter, *A. M. A. Arch. Neurol. Psychiatr.*, 64 (1950) 105.
21 G. Brante, *Acta Physiol. Scand.*, 18 (1949) 1, Supp. 63.
22 H. C. Agrawal, N. L. Banik, A. H. Bone, A. N. Davison, R. F. Mitchell and M. Spohn, *Biochem. J.*, 120 (1970) 635.
23 M. E. Smith, in R. Paoletti and D. Kritchevsky, *Advances in Lipid Research*, Vol. 5, Academic Press, New York, 1967, p. 241.
24 Y. Yaghiara, J. G. Salway and J. N. Hawthorne, *J. Neurochem.*, 19 (1969) 1133.
25 A. A. Abdel-Latif and L. G. Abood, *J. Neurochem.*, 13 (1966) 1189.

96 G. B. ANSELL AND S. SPANNER, *Biochem. J.*, 84 (1962) 12.
97 G. B. ANSELL AND S. SPANNER, *J. Neurochem.*, 15 (1968) 1371.
98 J. DOBBING, *Physiol. Rev.*, 41 (1961) 130.

Volatile Flavor of Strawberry Essence. I. Identification of the Carbonyls and Certain Low Boiling Subtances

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In aqueous distillates of Marshall strawberry puree, acetaldehyde, acetone, 2-hexenal, biacetyl, methanol, ethanol, and esters containing acetic and n-butyric acids were identified. An oil fraction separated by further distillation of the aqueous essence amounted to about 13% of the total essence and was about one-third free fatty acids: n-caproic, n-valeric, n-butyric, isobutyric, and acetic. The fatty-acid-free oil fraction seemed to contain all of the characteristic strawberry aroma. The amount of carbonyls and the total amount of essence as measured by carbon analysis were not correlated with the flavor potency of the essence, as judged by a taste panel.

A systematic study of the identities and amounts of the volatile flavoring compounds in strawberries is being made at the Western Utilization Research Branch. The purpose is to determine why some varieties of strawberries have more flavor than others. The method consists of identification and measurement of the volatile chemical compounds responsible for the quality, quantity, and potency of flavor. From this knowledge an objective chemical test may be developed for assaying the flavor potency. The volatile substances from fruit are a mixture of organic compounds varying in their amounts, solubility, and volatility. The low concentration of these volatile substances and the instability of the fruit increase the problems of separation and analysis. These organic substances also vary markedly in their flavor intensity.

Previous work by Coppens and Hoejenbos *(2)*, using an ether extract of 445 kg. of strawberry juice, showed the following compounds to be present: acetic acid, cinnamic acid, *n*-caproic acid, ethanol, biacetyl, ethyl acetate, iso-amyl alcohol, a formic ester, a butyric ester (probably ethyl), a caproic ester (ethyl or methyl), acetic esters, a benzoic ester, n-hexanol, ethyl salicylate, borneol, *α*-terpineol, terpin, and an unidentified acid and ketone. Other constituents reported as probably present were acetyl methyl carbinol, acetophenone, iso-fenchyl alcohol. McGlumphy *(6)* has confirmed acetophenone, biacetyl, cinnamic acid, and acetic acid. In addition he identified *n*-butyraldehyde, cis-terpinol hydrate, and probably ethyl acetoacetate.

MATERIALS AND METHODS

The 4 major steps in the procedure for this investigation are: (A) preparation of a puree or juice of the fruit, (B) removal

of the flavors from the puree or juice by distillation, (C) concentration of the dilute flavor solutions, and (D) performing appropriate analyses of the various constituents.

The equipment used for separating the volatile flavor from the fruit and for concentrating the essence solutions consists of a continuous laboratory vacuum flash evaporator which will remove 50% of the water from a puree or 70% from a juice in a single pass at about 100° F. (37.8° C.), and an all glass continuous bubble plate distillation column. This equipment was described in detail elsewhere *(3, 4)*. By means of this distillation column up to 2,000-fold essence solutions have been prepared.[a] Since the volatile constituents are organic, the recovery of essence was followed by means of a total carbon analysis of the various distillates *(1)*. Approimately 80% of the total strawberry volatiles are removed when 40% of the water is stripped from a puree, and practically no loss occurs in the concentration step with the still.

An analysis of the carbonyl content of the different varieties of berries was made on the aqueous concentrate mentioned above by a modification of the methods described by Lappin and Clark *(5)* and Neuberg and Strauss *(7)*. This method depends on the formation of the 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives of the carbonyl compounds, and the formation of an intense colored complex when potassium hydroxide is added to an alcoholic solution of these derivatives. Monocarbonyl derivatives form a red color, whereas dicarbonyl derivatives, such as biacetyl 2,4-DNPH, form a blue color.

The procedure is as follows: To each ml. of 40-fold essence is added 0.4 ml. of reagent (500 mg. of DNPH reagent in 50 ml. of 40% sulfuric acid) in a glass-stoppered bottle. This mixture is heated for 20 minutes on a steam bath and then cooled for one hour. The precipitated derivatives are then filtered on a Celite[b] coated sintered-glass funnel. The filter cake is washed with 2 or 3 ml. of water. The monocarbonyl derivatives which are soluble in alcohol are then washed from the funnel with about 20 ml. of hot alcohol and the filtrate is made up to 25 ml. with alcohol. The dicarbonyl derivatives are then washed from the funnel with a minimum of hot nitrobenzene and the filtrate is made up to 15 ml. with nitrobenzene. Nitrobenzene is the only good solvent known for this type of compound. Aliquots of the two filtrates are made up to 9 ml. with ethanol and one ml. of 2 N KOH is added. The intensity of the colors developed are estimated with a Klett-Summerson colorimeter and a 540 mμ (green) filter for the red solutions and a 590 mμ (orange) filter for the blue solutions. The amount of material is estimated from standard curves prepared from stock solutions of acetone and biacetyl 2,4-DNPH derivatives.

For analysis of the individual carbonyls, the alcohol-soluble 2,4-DNPH derivatives were separated with a silicic acid chromatographic column. Petroleum ether (b.p. 60° to 70° C., 140° to 158° F.)-ethyl ether 90/10 was used as the developing solvent. Three bands were separated and the material composing each band was identified as the 2,4-DNPH derivatives of 2-hexenal, acetone, and acetaldehyde. Identification was made on the assumption that with authentic samples there was no lowering of melting point and that ultraviolet absorption spectra and X-ray diffraction patterns were identical. The 2,4-DNPH derivatives of the carbonyls melted as follows: 2-hexenal 293 to 295° F. (145 to 146° C.), acetone 255° F. (124° C.), acetaldehyde 315° F. (157° C.), bis-biacetyl 615° F. (324° C.).

RESULTS AND DISCUSSION

The amounts of total carbonyls, acetone, 2-hexenal, and biacetyl are given in Table 1. The amount of acetaldehyde can be estimated as the difference between the total amount of carbonyls and those listed. There is no correlation between the total or individual carbonyl content of the essence and the flavor intensity.

A striking difference was found in the 2-hexenal content of the essence from frozen and unfrozen whole strawberries (Table 2). This aldehyde, sometimes called "leaf aldehyde," is apparently produced enzymatically by the fruit when pureed, as shown by the fact that either freezing or heating the whole berry will prevent the appearance of this aldehyde in the essence, while heating or freezing the puree from untreated whole berries will not affect the 2-hexenal content. Nye and Spoehr *(8)* have shown this compound to be produced in large amounts in crushed leaves of *Ailanthus glandulosa* (Chinese "tree of heaven"). Heating or crushing the leaves in the absence of oxygen prevented the formation of 2-hexenal. White *(11)* reported its presence in the essence from apple juice. The relative importance of 2-hexenal to the flavor of the fruit is yet to be determined.

TABLE 1

Carbonyl content and flavor intensity of the aqueous essence from various varieties of strawberries, 1948 season

Variety	Carbon	Carbonyls total	P.P.M. in puree			Flavor intensity
			Acetone	Biacetyl	2-hexenal	
Britemore	57	5.65	2.34	.82	trace	455
Sovereign	59	8.47	0.78	.76	trace	472
Marshall	75	2.6	1.56	.51	.038	630
Red Heart	55	19.2	8.55	1.30	trace	880
Corvallis	92	6.75	1.87	.59	trace	1164
President	84	11.9	2.02	1.48	trace	1300
Ettersburg	66	6.1	5.2	1.88	trace	2205

TABLE 2

2-hexenal content p.p.m. in frozen and unfrozen Marshall strawberries

Year	Frozen	Unfrozen
1948	0.038	.....
1949	0.066	7.2
1950	.....	6.1

On the basis of results thus far, neither total essence as measured by carbon analysis nor carbonyl content seemed related to the flavor strength of the berries. Therefore, it became desirable to investigate other chemical components of the essence. Since freezing was shown to cause alterations, fresh Marshall berries were used for a systematic general analysis of the essence.

Eight hundred pounds of strawberries obtained from the State of Washington were cleaned, stemmed, pureed, and processed immediately. The stripped essence from the puree was concentrated 850-fold. Total-carbon and carbonyl analyses were made on a 50-fold essence, since separation of an oil occurred when the concentration reached about 300-fold.

The major portion of the organic volatiles was separated from the 850-fold product by distillation in a fraction which boiled below 190° F. (88° C.). This fraction contained no typical strawberry aroma. The remainder of the essence was collected with a large amount of water in a fraction boiling between 203° and 212° F. (95° and 100° C.). This fraction contained a

[a] The word fold as used here means essentially the extent of concentration. In this example it would take 2,000 g. of puree to make 1 g. of 2,000-fold essence.

[b] Mention of specific products does not constitute endorsement by the Department of Agriculture over others of a similar nature not mentioned.

small amount of a water-insoluble oil and possessed a powerful aroma of strawberries. The organic components of this fraction were separated from the water by an isopentane extraction and appeared as a light-yellow oil after removal of the isopentane.

An over-all analysis of the strawberry essence is shown in Table 3. The amount of essence is shown as parts per million of carbon and is also calculated as ethyl alcohol. The amount of the 4 carbonyl compounds mentioned before — acetaldehyde, acetone, 2-hexenal, and biacetyl—is shown, and their percentages of total

TABLE 3
Analysis of fresh Marshall strawberries

Description	P.P.M. in original puree	Percent of total essence
Essence (as carbon)	42	100
Essence (as ethanol)	80	100
Acetaldehyde	4.9	6.1
2-Hexenal	7.2	9.0
Acetone	2.7	3.4
Biacetyl	0.21	0.26
Ethanol	45.5	57.0
Methanol	4.7	5.8
Esters (calc. as ethyl acetate)	9.4	11.7
Acids of esters: acetic, *n*-butyric		
Water Insoluble Oil	7.5	9.5
Free Fatty Acids	3.00	3.7
n-caproic	1.5	1.9
n-valeric	0.8	1.0
n-butyric-isobutyric	0.52	0.7
Acetic	0.12	0.15

essence are listed. Two alcohols, methanol and ethanol, were separated by distillation and measured. They were identified by boiling point and mixed melting point of their 3,5-dinitrobenzoate derivatives, which showed no depression with authentic samples: ethanol 3,5-DNB (m.p. 92° C.) methanol, 3,5-DNB (m.p. 106° C.) As may be seen, ethanol accounts for more than half of the total essence.

The amounts of esters calculated as ethyl acetate were determined by saponification of the low-boiling fraction. Only acetic and *n*-butyric acids were found in this ester group. The organic acids were separated by the chromatographic procedure described by Ramsey and Patterson *(9, 10)* and identified by X-ray diffraction pattern of their crystalline sodium salts. This method of identifying the acids was found to be very satisfactory, provided care was used in drying the samples. All of the sodium salts were dried at 20% relative humidity and were transferred to the capillaries in a dry box. The iso-acids of butyric, valeric, and caproic were found to crystallize with one molecule of water under these conditions.

The highly flavored oil fraction amounting to about 13% of the total essence was about one-third free fatty acids: *n*-caproic, *n*-valeric, *n*-butyric, isobutyric, and acetic. These acids, in the concentration found in the fruit, cannot be detected by taste in distilled water. The remainder of the oil fraction, although amounting to only about 7.5 p.p.m. in the fruit, has such a powerful flavor intensity that the aroma of strawberry can be clearly recognized in a concentration of 0.1 p.p.m. This is about one-tenth of a gram per ton of fruit. The composition of this oil is presently under investigation.

SUMMARY

Different varieties of strawberries vary in their flavor intensity. The amount of total organic volatiles does not measure the strength of flavor, nor does the carbonyl content seem related to flavor intensity. Freezing or heating the whole fruit reduces the 2-hexenal content of the essence to a trace. In all the volatile organic compounds called essence we have a major portion (90% or greater) which in themselves are not reminiscent of strawberries. The typical strawberry aroma appears to reside in an oil fraction amounting to about 7% of the total essence. It may turn out, however, that the well-balanced flavor will require the presence of these less aromatic constituents. This will have to be determined after a complete analysis of the oil has been made.

LITERATURE CITED

1. Christensen, B. E., Wong, R., and Facer, F. J. Determination of the carbon content of organic materials. *Ind. Eng. Chem., Anal. Ed.*, **12**, 394 (1940).
2. Coppens, A., and Hoejenbos, L. Volatile constituents of strawberry juice. *Rec. Trav. Chim.*, **58**, 680 (1939).
3. Dimick, K. P., and Makower, B. A laboratory-scale continuous vacuum flash evaporator. *Food Technol.*, **5**, 517 (1951).
4. Dimick, K. P., and Simone, M. J. A laboratory continuous distillation column for concentration of aqueous solutions of volatile flavors. *Ind. Eng. Chem.*, **44**, 2487 (1952).
5. Lappin, G. R., and Clark, L. C. Colorimetric method for determination of traces of carbonyl compounds. *Anal. Chem.*, **23**, 541 (1951).
6. McGlumphy, J. H. Fruit flavors. *Food Technol.*, **5**, 353 (1951).
7. Neuberg, C., and Strauss, E. Microdetermination of dicarbonyl compounds on the coloring of nitro compounds in alkaline solutions. *Arch. Biochem.*, **7**, 211 (1945).
8. Nye, W., and Seifter, H. A. The isolation of hexenal from leaves. *Arch. Biochem.*, **2**, 23 (1943).
9. Ramsey, L. L., and Patterson, W. I. Separation and identification of the volatile saturated fatty acids (C_1 – C_4). *J. Assoc. Offic. Agr. Chem.*, **28**, 644 (1945).
10. Ramsey, L. L., and Patterson, W. I. Separation and determination of the straight-chain saturated fatty acids C to C_6 by partition chromatography. *J. Assoc. Offic. Agr. Chem.*, **31**, 139 (1948).
11. White, J. W., Jr. Composition of a volatile fraction of apples. *Food Research*, **15** (1) 68 (1950).

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EFFECTS OF ENERGY DONORS, METABOLIC INHIBITORS AND SUBSTRATES ON CAROTID CHEMORECEPTOR ACTIVITY[1]

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The injection of substances into the common carotid artery is a commonly employed method for assaying their excitant or depressant properties on the chemoreceptors of the carotid body. The number of such substances known to affect the chemoreceptor activity is steadily increasing. In this study we have examined the action of several agents, which are known to have varied effects on cellular metabolism. These agents included high-energy bond phosphate derivatives, various substrates of intermediary carbohydrate metabolism and some inhibitors of intermediary metabolism.

METHODS. Fifteen cats anesthetized with chloralose (60 mgm./kgm.) and urethane (250 mgm./kgm.) were used in this study. All of the animals were artificially respired with room air to the extent that spontaneous respiratory movements were barely abolished. The right carotid sinus nerve was dissected out and after removal of its sheath was used for recording action potentials according to classical methods. Pure chemoreceptor preparations were obtained in some experiments by cutting most of the nerve branches from the carotid wall and compressing the sinus wall thereafter. Blood pressure from one of the two major branches of the external carotid artery was recorded through a Statham P23A gauge and amplifier on the second beam of a Dumont dual beam cathode ray oscillograph; the other branches of the carotid arterial system were ligated. All of the substances injected were diluted with saline and injected in a constant volume of 0.5 ml. at a constant speed (duration of injection 3.0 seconds) into the lingual artery through a fine polyethylene catheter. The tip of the catheter usually lay at the orifice of the origin of the lingual artery from the common carotid artery. With the catheter in this position the latent periods of chemoreceptor excitation, obtained after the injection of stimulants, were shorter than latent periods determined previously (Dontas and Nickerson, in press) with the catheter tip placed 1.0 to 1.5 cm. into the lingual artery. The response of the chemoreceptors of the opposite carotid body was examined in several experiments; for this purpose, injections were given into the *left* carotid artery, while the activity of the *right* carotid nerve was followed, as described above. With this method *right* chemoreceptor responses occurred only after large doses of stimulants into the left carotid area and after significantly longer latent periods.

RESULTS. 1. *Energy-rich phosphates.* Sodium adenosine triphosphate (ATP), adenosine monophosphate (AMP) and pyrophosphate all exerted a moderate to strong chemoreceptor excitation, which *normally* had a latent period of 7 to 9 seconds with concentrations of the tested agents of 100 microgm./ml. or higher

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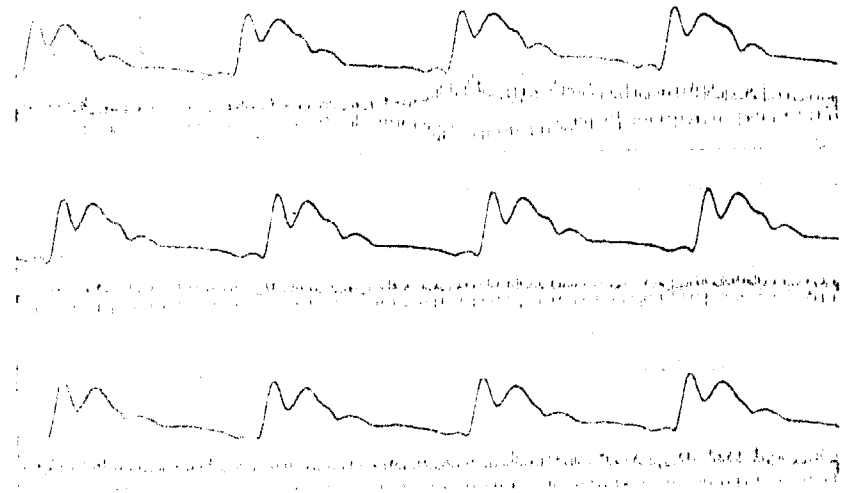

FIG. 1. In each strip from above downward: timer (1/50 sec.), carotid pulse and action potentials of carotid nerve (all pressoreceptors cut). Mild chemoreceptor activity, animal barely overventilated. Upper strip: at interruption of timer beginning of intracarotid injection of 100 microgm. ATP (in 0.5 ml.). Strong chemoreceptor discharge with latent period of 0.3 second. Middle: 1.2 seconds later, at end of injection; chemoreceptor activity back to normal. Lower: 9 seconds after A; "late" chemoreceptor stimulation. Blood pressure was 140 mm. Hg throughout. Calibration in upper strip: 50 microvolts.

(0.16 micromols/ml., 0.26 micromols/ml. and 0.22 micromols/ml. for ATP, AMP, and pyrophosphate, respectively). The response was greater with ATP than with the other two agents, and its onset tended to occur progressively earlier as the injections were repeated, especially with larger doses. Only ATP (fig. 1) displayed a clearcut "early" response (latent period shorter than 2 seconds) similar to that shown previously for potassium ions, lobeline and acetylcholine (Dontas and Nickerson, in press). Even seemingly maximal excitatory doses of AMP or sodium pyrophosphate, leading to chemoreceptor discharge lasting for several minutes, did not evoke an "early" chemoreceptor response (fig. 2). This "early" response could be obtained either with single large doses of ATP (concentrations of 1 mgm./ml.) or with smaller doses of ATP (50 microgm./ml.) after repeated injections of the three agents. The "late" chemoreceptor response was of a slowly progressing and regressing type and lasted for 10 to 20 seconds with concentrations of the three agents of the order of 0.2 to 0.4 micromols/ml. A period of depression sometimes preceded the excitation, and was somewhat longer than the nonspecific depression occurring 2 to 3 seconds after the beginning of the injection.

Monosodium phosphate ($NaH_2PO_4 \cdot H_2O$) also led to mild late excitation, but with enormously higher doses (20 micromols/ml.), indicating that the strong excitatory action of the other compounds might be due to their energy-rich bond content.

All of the phosphate compounds tested led to a continuous (not pulse syn-

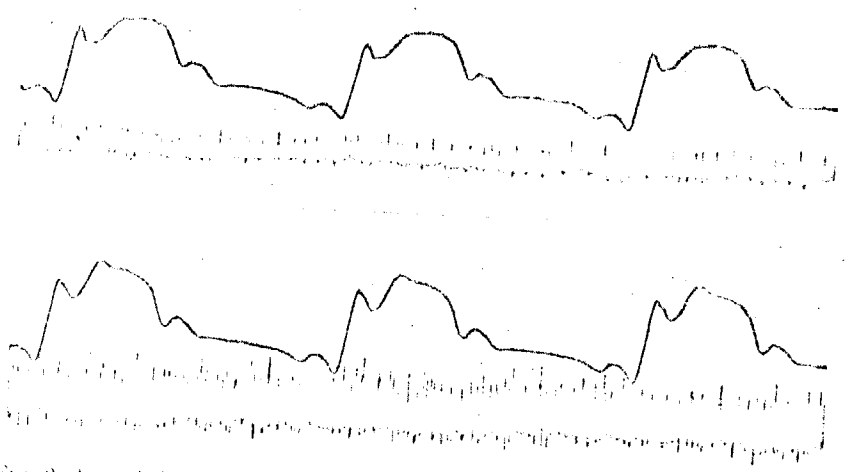

FIG. 2. Animal the same as in fig. 1. Above: beginning of intracarotid injection of 2.5 mgm. sodium pyrophosphate. No "early" response. Blood pressure 172 mm. Hg. Below: three minutes later; very intense chemoreceptor response. Blood pressure 180 mm. Hg. A minimal increase of discharge in this case appeared first at 3.4 seconds and disappeared 1.4 seconds after the beginning of the injection; the recorded secondary, much stronger, discharge appeared subsequently at 45 seconds and lasted about 20 minutes.

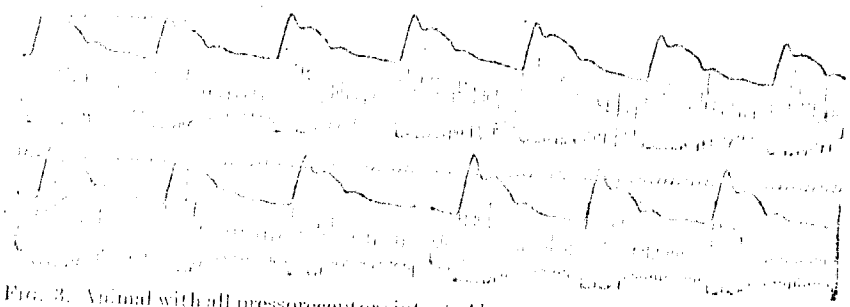

FIG. 3. Animal with all pressoreceptors intact. Above: at interruption of timer beginning of intracarotid injection of 100 microgm. sodium pyrophosphate. Increase of discharge of medium and large pressoreceptor fibers; no change in the activity of the tonically discharging fiber (pressoreceptor). Below left: 7 seconds later; chemoreceptor discharge. Right: 10 seconds after V: return to normal of chemoreceptor discharge and marked increase of the rhythmicity of the tonically active pressoreceptor. Blood pressure was 130 mm. Hg throughout.

chronous) repetitive activity of medium voltage pressoreceptor spikes, which appeared some seconds after the end of the injection. This increase should not be confused with the normally obtained enhanced pressoreceptor discharge *during* the injection, *seen* with any injection; the latter is pulse related (fig. 3) and usually returns to normal immediately after the end of the injection.

2. *Inhibitors of oxygen consumption and uncouplers of oxidative phosphorylation.*

Sodium azide in concentrations of 0.5 to 2 × 0.16 micromols/ml. had a definite and long-lasting excitant action, qualitatively indistinguishable from that of sodium cyanide; the onset occurred 6 to 8 seconds after the beginning of the injection and the discharge, depending upon the dose, could last up to one minute. Cyanide and azide, both inhibitors of cytochrome oxidase, had a competitive effect, in that each blocked the excitatory effect of the other for some minutes. Small doses of azide depressed cyanide response, without affecting that to acetylcholine; larger doses, however, were followed by a long-lasting, and sometimes irreversible, depression of chemoreceptors, nonresponsive to any stimulant. Azide had, as described previously with cyanide (Dontas, 1954), a moderate stimulating effect on pressoreceptors, which fired repetitively after its injection for periods up to 30 seconds.

2,4-Dinitrophenol in concentrations of 100 to 1000 microgm./ml. resulted also in strong and long-lasting chemoreceptor excitation (fig. 4); this confirms previous findings by the Swedish group (Jarisch *et al.*, 1952). The response appeared earlier with larger doses and the shortest observed latency was that of 3.2 seconds. The response of chemoreceptors to cholinergic and anoxic stimuli was normal following dinitrophenol administration.

We tested three barbiturates, all potent uncouplers of oxidative phosphorylation. Thiopental was tested in concentrations of 2 to 10 mgm./ml., pentobarbital in the same doses and the convulsant barbiturate, 5-(1-3-dimethylbutyl)-5-ethyl sodium barbiturate), was injected in concentrations of 0.5 to 2 mgm./ml.

Thiopental markedly depressed chemoreceptor activity for periods of 0.5 to 3 minutes, usually preceding and overlapping with the hypotension (fig. 5). During the blood pressure recovery, chemoreceptor activity returned to normal and on

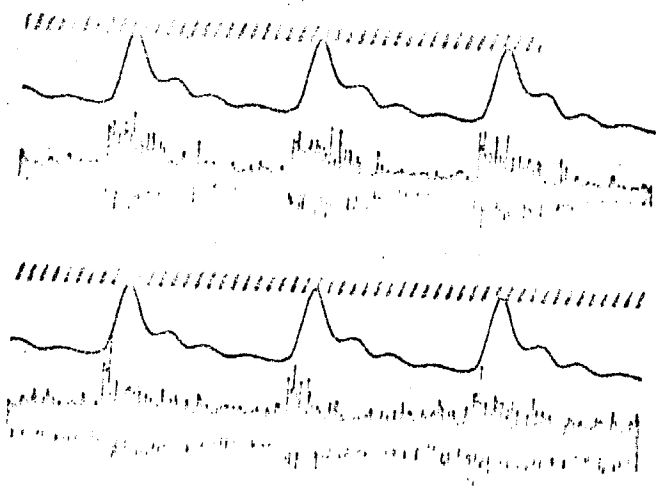

FIG. 4. Above: control and beginning of intra-arterial injection of 100 microgm. 2,4-dinitrophenol. Below: 5 seconds later. Intense chemoreceptor activity. Blood pressure, unchanged, 150 mm. Hg.

occasion, with the animals just adequately ventilated, a slight rebound activity became apparent. The findings with pentobarbital were similar.

The other barbiturate tested (differing from pentobarbital by the presence of an extra methyl group in the butyl chain) produced no hypotension following its intra arterial administration. Convulsive, but also definitely subconvulsive, doses were followed by heavy chemoreceptor discharge, occurring with a latency of 8 to 12 seconds and lasting for 1 to 2 minutes (fig. 6). Blood pressure usually increased somewhat during this period, probably because of the increased vasoconstrictor outflow elicited by the general chemoreceptor activation.

3. *Substrates.* Glucose and the sodium salts of succinic, acetic and pyruvic acid were given in total doses of 1 to 10 mgm. 0.5 ml.

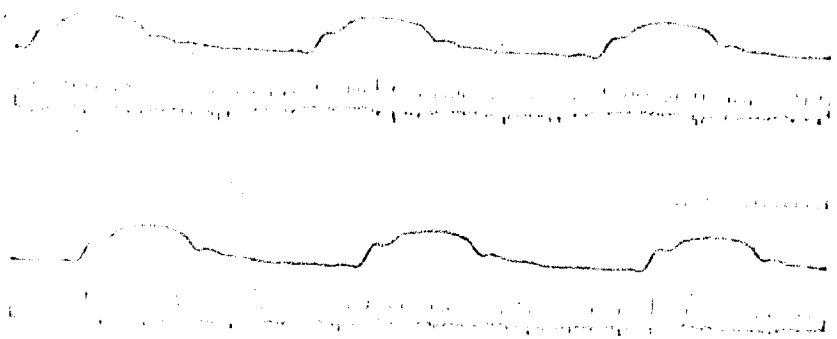

FIG. 5. Pure chemoreceptor preparation. Above: control and beginning of intra-arterial injection of 10 mgm. thiopental. Blood pressure 190 mm. Hg. Below: 30 seconds later, marked decrease of chemoreceptor activity. Blood pressure 190 mm. Hg.

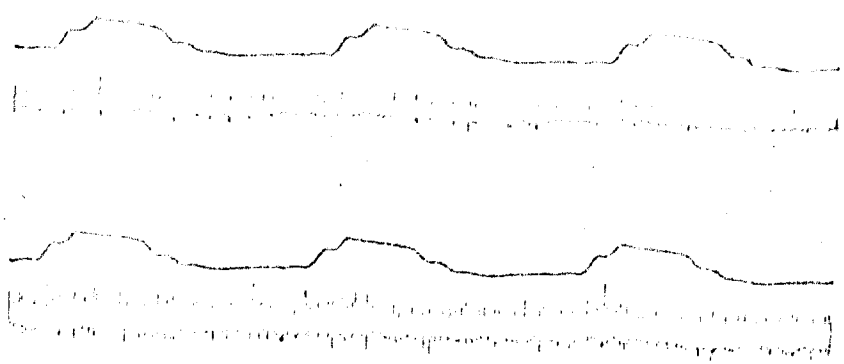

FIG. 6. Animal same as that of figure 5. Above: control and beginning of intra arterial injection of 1 mgm. of the convulsant barbiturate. Below: 60 seconds later, intense chemoreceptor discharge. Blood pressure 195 mm. Hg in both strips.

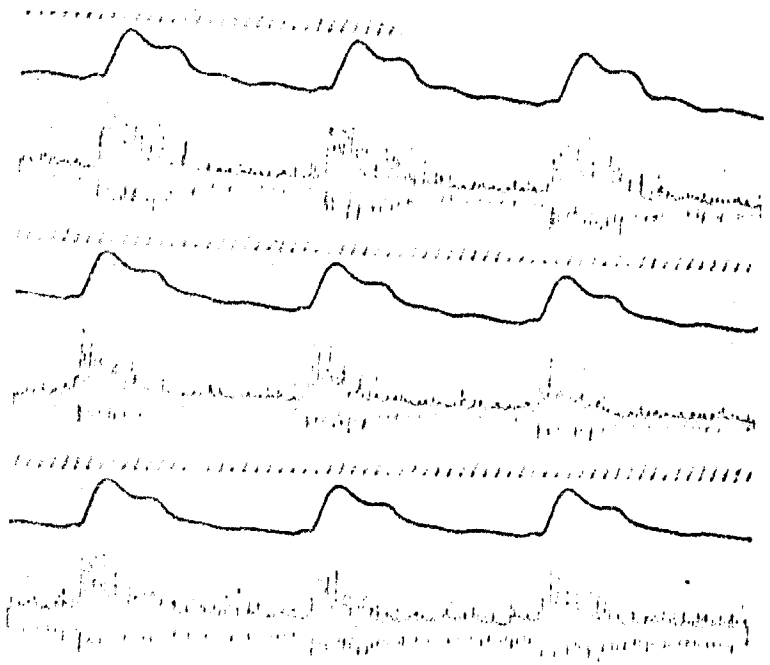

FIG. 7. Upper: control and beginning of intra arterial injection of 2 mgm. sodium succinate. Middle: 5.8 seconds later, decreased chemoreceptor activity. Lower: 11 seconds after A: "rebound" of chemoreceptor activity. Blood pressure 145 mm. Hg throughout.

Chemoreceptor activity decreased with all substances tested; this reduction appeared within 2 to 4 seconds, with the doses employed, and lasted 8 to 10 seconds. Following this, a moderate but constant mild chemoreceptor excitation appeared lasting for considerable periods (20 to 40 seconds).

The early depression was slightly more prolonged than control injections of line of the same temperature, and seemed, therefore, to represent a specific effect of these substances. The late excitatory effect of the substrates was also more pronounced than similar "rebounds" from inactivity following saline injections and also lasted for considerably longer periods of time. Higher concentrations of the injected substrates did not elicit stronger or more prolonged chemoreceptor discharge and maximal effects were obtained by concentrations 1 to 3 mgm. 0.5 ml. glucose saline. The various substrates tested had approximately the same potency and duration of action (fig. 7).

As the solutions of the substrates were prepared in isotonic saline and the resultant osmolarity thus exceeded somewhat that of plasma, we gave in some animals injections of isotonic, hypotonic and hypertonic glucose and sucrose prepared in distilled water. A surprising finding was that small amounts of sucrose increased chemoreceptor activity just as glucose did, and that both substances displayed the initial long lasting depression. Differences in osmolarity did not seem to affect chemoreceptor activity.

DISCUSSION. In discussing these findings one should bear in mind the circum-

stances under which chemoreceptor discharge occurs normally. The normal stimulus to the carotid body is probably oxygen lack; this can be induced in the laboratory animals by administration of oxygen poor mixtures or injections of cyanide. The inhibition of terminal oxidation by cyanide leads to accumulation of pyruvate, lactate and phosphates of the triose and hexose level and a shift of the intracellular pH toward the acid side. The accumulation of these metabolites requires some time and the effects of cyanide therefore occur with considerable delay after the injection. Acetylcholine, thought by several workers to be the true "transmitter" (Lauderen *et al.*, 1952), can be involved in this scheme only if released by the intracellular anoxia and must act within the cell on the neurotubule ending "freely" there.

Metabolic inhibitors acting in the same point in carbohydrate metabolism as cyanide should mimic the effects of the latter; such is the case of azide, which has a competitive action with cyanide, so that the first injected substance prevents chemoreceptor excitation induced by the other, injected subsequently. The effects of azide (in concentrations of the order of 0.5 mgm./ml.) are longer lasting, probably on account of its slow inactivation compared with the relatively rapid removal of cyanide. With relatively large doses of sodium azide, one is able to dissociate between cholinergic and anoxic stimulation of the chemoreceptors, i.e. chemoreceptor responses to anoxia (NaCN) are inhibited for some time whereas cholinergic agents still elicit good responses. Similar findings were obtained by A. Euler *et al.* (1939) with intravenous ammonia. As pointed out, however, these are quantitative differences, as chemoreceptors quickly become unresponsive to any stimulus. These findings indicate that depression of metabolism and accumulation of products of carbohydrate breakdown between the glucose and pyruvate level is associated with enhanced chemoreceptor activity.

Conversely, injection of glucose or other substrates, in the absence of any enzyme inhibition led to an initial long-lasting depression. Substrate forcing should not enhance chemoreceptor metabolism and thus depress chemoreceptor discharge, as long as the rate of enzymatic reaction is not limited by the amount of substrate present. The late rebound, following the initial chemoreceptor depression may be the result of accumulation of products of intermediary metabolism, but the persistence of the excitation following injections of sucrose (resembling the effect of sucrose on papillary muscle contractility, Chenoweth, unpublished) makes speculations based on disturbed metabolism rather hazardous.

Still less clear are the actions of the energy-rich phosphates. All of these substances displayed strong long-lasting excitatory properties. It is not known, however, whether these substances can penetrate the cell membrane and if therefore their action is extra- or intracellular. The extremely short latency of the initial ATP response (shorter even than the "early" acetylcholine response) could be compatible with a surface effect, similar to the ATP action on other receptors (Feldberg and Feldberg, 1948). The late responses of both ATP and the other phosphates tested might be due to their slow intracellular diffusion and action (possibly on acetylcholine synthesis), or to transformation of these

compounds to ATP and a direct (?) action of the latter. It has been clearly shown, however, that phosphates containing many high-energy bonds are more potent chemoreceptor stimulants and therefore the chemoreceptor response is in some way dependent upon the amount of available high-energy bond.

Finally, a word about the agents dissociating phosphorylation from oxidation. Such agents have been called "uncouplers" as they depress energy yielding reactions (phosphorylation) without (usually) affecting the normal cellular oxygen consumption. All the agents tested, i.e. dinitrophenol, the convulsant barbiturate, thiopental and pentobarbital are reported to be potent uncouplers (Brody and Bain, 1951). It was hoped that chemoreceptors would be a system where these agents would behave similarly. Unfortunately the action of these agents was not uniform; dinitrophenol and the convulsant barbiturate excited, whereas thiopental and pentobarbital depressed chemoreceptor activity. Thus, it is unlikely that the depression of oxidative phosphorylation is the factor influencing chemoreceptor activity.

In conclusion, the above experiments added several new substances to the array of the known chemoreceptor excitants and permit some further insight in the behavior of metabolic activity of chemoreceptors. They do not provide an answer, however, to the controversial issue of the cholinergic nature of chemoreceptor excitation and it appears unlikely that experiments, other than perfusion studies, will yield "conclusive" proof of this theory.

SUMMARY

Carotid chemoreceptor activity was studied electrically, as affected by the intracarotid injection of agents related to carbohydrate metabolism.

All high-energy phosphates tested (ATP, AMP and pyrophosphate) excited chemoreceptors after a variable latent period (concentrations of 0.2 micromols/ml. or higher). ATP alone displayed a strong "early" as well as the variable "late" chemoreceptor excitatory action. NaH_2PO_4 enhanced activity only if given in enormously higher concentrations (20 micromols/ml.).

Azide displayed excitatory action similar to (and competitive to) cyanide; selective inhibition of anoxic stimuli with relatively unchanged responsiveness to cholinergic ones was present after moderate amounts of azide; large doses were followed by complete unresponsiveness to any stimulus.

Thiopental and pentobarbital depressed, whereas a convulsant barbiturate and 2,4-dinitrophenol enhanced chemoreceptor activity.

Glucose and several other products of carbohydrate metabolism depressed chemoreceptor activity for periods up to 10 seconds, and a moderate rebound, lasting several seconds, was observed thereafter. The late chemoreceptor rebound was less marked with high concentrations of substrates and both responses were present following injections of sucrose (non-metabolized).

The results are analyzed on the basis of current concepts of chemoreceptor excitation and with regard to the hypothesis of depression of oxidative phosphorylation as a stimulus for chemoreceptor activation. The data show that

several excitatory mechanisms may operate and that "uncoupling" agents may have diametrically opposite action on these receptors.

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REFERENCES

BRODY, T. M., AND BAIN, J. A.: THIS JOURNAL, 110: 148, 1954.
DONTAS, A. S.: Fed. Proc., 13: 37, 1954.
EMMELIN, N., AND FELDBERG, W.: Brit. J. Pharmacol., 3: 273, 1948.
EULER, U. S. VON, LILJESTRAND, G., AND ZOTTERMAN, Y.: Scand. Archiv Physiol., 83: 132, 1939.
JARISCH, A., LANDGREN, S., NEIL, E., AND ZOTTERMAN, Y.: Acta Physiol. Scand., 25: 195, 1952.
LANDGREN, S., LILJESTRAND, G., AND ZOTTERMAN, Y.: Acta Physiol. Scand., 26: 264, 1952.

Inhibition of Appetite in Dairy Cattle by Certain Intermediate Metabolites

MANNING *et al.*[1] have discussed the applicability of the glucostatic theory of Mayer *et al.*[2] to the regulation of food intake in sheep. Glucose in ruminant animals plays a relatively minor part in energy metabolism, since a major portion of ingested carbohydrates are degraded to volatile fatty acids which are absorbed into the blood stream via the rumen wall[3]. Consequently, a chemoreceptor mechanism of regulation of intake in ruminants may depend on any of several metabolites. The objective of this experiment was to examine several major intermediate metabolites for their effect on appetite.

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

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

The administration of the higher level of acetic acid and sodium acetate resulted in a highly significant reduction in feed intake during the 8-hr. injection period; with both metabolites consumption was negligible. Consumption during the other 16 hr. of the three injection days approached normal. However, the 24-hr. feed-intake values during the three days were significantly lower for the treatment twins than for the control twins. Administration of propionic acid resulted in significant reductions in intake during the 8-hr. and 24-hr. periods. There was very little carry-over effect from acetate, acetic or propionic acid injections, since eating began very soon after cessation of each 8-hr. injection period.

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

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

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

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

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

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

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[1] Manning, R., Alexander, G. I., Krueger, H. M., and Pegram, R., *Amer. J. Vet. Res.*, **20**, 242 (1959).

[2] Mayer, J., and Bates, M. W., *Amer. J. Physiol.*, **168**, 812 (1952).

[3] Barcroft, J., McAnally, R. A., and Phillipson, A. T., *J. Exp. Biol.*, **20**, 120 (1944).

[4] National Research Council Publication 464 (1956) gives daily maintenance requirement of a 1,000-lb. cow as 14·7 therms of digestible energy; adjustments for variation in bodyweight were proportional to 0·75 log bodyweight.

[5] Vallance, W. S., and McClymont, G. L., *Nature*, **183**, 140 (1959).

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THE EFFECT OF ORGANIC ACIDS ON MAMMALIAN TUBERCLE BACILLI

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Histological studies have revealed that active tuberculous lesions often contain surprisingly few acid-fast organisms (1-3). In caseous areas, bacilli detectable by the Ziehl-Neelsen technique are seen chiefly at the periphery of the lesion but only in very small numbers in its central part, and there is evidence that the bacilli become more and more scarce in the aging lesion as long as it remains closed. These findings are rendered the more surprising by the fact that caseous material often becomes the seat of enormous bacterial multiplication when the lesion opens into a blood vessel or a bronchus. Since the classical processes of immunity—humoral or cellular—can hardly account for the progressive disappearance of the acid-fast organisms from closed necrotic areas, it seems necessary to postulate that inflammation and necrosis bring about a physicochemical environment unfavorable to the survival and multiplication of tubercle bacilli.

Inadequate aeration is the most obvious of the factors that might play a role in the progressive sterilization of closed tuberculous lesions, for it is known that —*in vitro* at least—tubercle bacilli are dependent on aerobic metabolism for their survival as well as for the efficient performance of their synthetic processes. *In vivo* findings are also compatible with this view. Thus, the fetus of the guinea pig is markedly resistant to tuberculous infection—a property that may be due to the low oxygen content of the fetal blood (4). Similarly, the bacilli do not do well in the collapsed areas of the lung where blood flow is diminished (5), and it has been shown that guinea pigs housed in chambers under reduced oxygen tension are more resistant to experimental tuberculous infection than when living at ordinary atmospheric pressure (6, 7). It is probable, however, that any oxygen pressure compatible with the life of adult animal cells will be adequate, not only for the survival of tubercle bacilli, but also for their multiplication—even though at a slow rate. Furthermore, some oxygen must reach the caseous material by diffusion and be present in solution in its aqueous phase. It appears unlikely, therefore, that deprivation of oxygen during life can be sufficiently complete to be the direct and only cause of the progressive disappearance of bacteria from the lesion.

Decreased blood flow and low oxygen tension naturally have many indirect

biochemical effects. For example, increased production of lactic acid resulting from semi-anaerobic metabolism of tissue cells, and slower removal or neutralization of carbonic acid, will alter the normal acid-base relationships in body fluids and tissues and may, in certain cases, overcome their buffering power. In fact, it is known that areas of inflammation and caseation can become markedly acidic (approximately pH 6.5), at least during certain phases of their evolution, and it has been claimed that they contain more lactic acid than do normal tissues (8-12). But it is unlikely that the hydrogen ion concentration at the site of the tuberculous lesion can ever become high enough to be the primary cause of toxic effect on acid-fast bacilli since the optimal range for their multiplication *in vitro* is between pH 6.2 and 6.8.

Another characteristic of inflammatory and caseous areas is the accumulation at their site of a variety of metabolic constituents and of products resulting from autolytic breakdown of tissue cells. Several of these have recently been found in our laboratory to possess marked bacteriostatic activity against tubercle bacilli. The present paper is concerned with the effect on the growth of these organisms of some of the organic acids produced during cellular metabolism or by autolytic changes.

EXPERIMENTAL

Materials and Methods.—The experiments to be described were carried out with twelve different cultures of mammalian tubercle bacilli exhibiting different degrees of virulence. The results obtained were of the same order with all strains tested, except for the fact to be discussed in a subsequent publication that most of the attenuated strains were more susceptible than the fully virulent strains to the bacteriostatic effect of organic acids. For the sake of brevity, only the results obtained with the following two fully virulent strains will be reported in the present publication.

H37Rv—a strain of human origin obtained from Mr. W. Steenken, Jr., of the Trudeau Sanatorium.

Ravenel—a bovine strain obtained from Dr. J. Freund of the Public Health Research Institute of the City of New York.

These cultures have been maintained for the past 4 years by transfer in liquid Tween-albumin medium at intervals of 1 to 3 weeks.

Determination of the effect of organic acids on growth was carried out in liquid and agar media of the semisynthetic general type described in earlier publications (13, 14). Two basal media were used, one containing as source of nitrogen 0.2 per cent asparagine and 0.05 per cent of enzymatic hydrolysate of casein; another in which 0.2 per cent sodium glutamate was substituted for asparagine. Tween 80, oleic acid, and glucose were added or omitted as indicated in the text. Bovine plasma fraction V (in solution in 0.9 per cent NaCl) or defibrinated filtered bovine serum served as source of albumin. They were sterilized by filtration and were added aseptically to the sterile media. The reaction of the different components of the medium was adjusted to the desired pH with NaOH or HCl prior to mixing.

The organic acids to be tested were added, in the form of their sodium salt, in the proper concentration to the medium before autoclaving.

With the exceptions noted below, they were all C.P. grade, obtained from the Amend Drug and Chemical Company, Inc. They were not further purified. Oleic and linoleic acids were Technical grade.

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Lignoceric acid was obtained from the collection of the late Dr. P. A. Levene. It was prepared from egg yolk sphingomyelin and listed as lot No. 1097 in the collection.

In the case of lactic acid, most experiments were carried out with Merck "lactic acid reagent." In a few confirmatory tests (not recorded in the present paper), there was used a preparation of the Zn salt of *l*(+)-lactic acid prepared by Dr. Thompson of the Chemistry Department of Ohio State University and obtained through the courtesy of Dr. Lippman H. Geronimus.

The Effect of Different Organic Acids on the Multiplication of Tubercle Bacilli in vitro.—

Experiment I.—Test tubes 25 mm. in diameter received 5 cc. of asparagine basal medium at pH 6.5, containing 0.02 per cent Tween 80. The sodium salts of the various organic acids to be tested were added in 0.5 cc. volume of solutions varying in concentration by twofold steps as indicated in Table I. The tubes were inoculated by the addition of 0.5 cc. of either one of the two following culture dilutions.—

(*a*) 5 cc. of 10-day-old culture in Tween-albumin medium added to 45 cc. of a 5 per cent bovine albumin solution in physiological saline, containing 0.05 per cent neutralized oleic acid (designated as oleic acid-albumin complex in reference 14).

(*b*) 5 cc. of 10-day old culture in Tween albumin medium added to 40 cc. of 5 per cent bovine albumin solution and 5 cc. of 50 per cent glucose solution (giving a final concentration of 0.5 per cent glucose in the culture medium, but no oleic acid).

The cultures were incubated at 37°C. and the extent of growth recorded after 1 week's and 2 weeks' incubation (Table I).

In confirmation of the results of earlier studies (15, 16), the data presented in Table I illustrate the dual effect of oleic acid on tubercle bacilli. When added to the medium in low concentration and in admixture with the proper amount of serum albumin (0.005 per cent oleic acid, 0.5 per cent albumin), oleic acid markedly enhanced bacterial growth. On the other hand, it inhibited growth when present in higher concentration in the culture medium. Unexpected was the finding that, like oleic acid, the shorter chain aliphatic acids and lactic acid did also exert an inhibitory effect on growth.

In all cases the concentrations of organic acid required for inhibition of growth were smaller in the oleic acid-albumin medium than in the glucose-albumin medium, although the former gave more abundant growth than the latter in the absence of inhibitory substances. The interpretation of this finding will be considered later in the discussion.

In the experiment just described, the growth inhibitory effect occurred in the presence of an agent, Tween 80, which wets the surface of tubercle bacilli and causes them to grow submerged and, therefore, in a semi-anaerobic state. In the following experiment, the effect of the organic acids was tested under conditions in which the bacterial surface remained hydrophic (in media containing oleic acid-albumin complex without Tween) and growth took place on aerobic agar surfaces as well as in submerged cultures in liquid media.

TABLE I

The Inhibitory Effect of Organic Acids on the Growth of Tubercle Bacilli in Liquid Tween-Albumin Media

Medium pH 6.5	Final concentration of organic acid		Growth after incubation at 37°C. for			
			1 wk.		2 wks.	
			H37Rv	Ravenel	H37Rv	Ravenel
		per cent				
Asparagine medium with oleic acid-albumin complex (no glucose)	Acetic acid	0.2	−	−	−	−
	" "	0.1	−	−	−	−
	" "	0.05	−	+	+	++++
	Butyric "	0.04	−	−	−	−
	" "	0.02	−	+	−	++++
	" "	0.01	−	+++	+	++++
	Capric "	0.01	−	−	−	−
	" "	0.005	−	−	−	−
	" "	0.0025	++	++	++++	++++
	Oleic "	0.01	−	−	−	−
	" "	0.005	+++	−	+++++	−
	" "	0.0025	+++++	++	+++++	+++++
	Lactic "	0.2	−	−	−	−
	" "	0.1	++	++	++++	++++
	0	0	+++++	+++++	+++++	+++++
Asparagine medium with 0.5 per cent glucose and albumin (no oleic acid)	Acetic acid	0.2	−	−	−	−
	" "	0.1	−	++	−	+++
	" "	0.5	+++	+++	+++	+++
	Butyric "	0.04	−	−	−	−
	" "	0.02	+++	+++	+++	+++
	" "	0.01	+++	+++	+++	+++
	Capric "	0.01	−	−	−	−
	" "	0.005	−	++	−	+++
	" "	0.0025	+	+++	+++	+++
	Oleic "	0.01	−	−	−	−
	" "	0.005	+++++	++++	+++++	++++++
	" "	0.0025	++++++	++++	+++++	+++++
	Lactic "	0.2	+++	+++	+++	+++
	" "	0.1	+++	+++	+++	+++
	0	0	+++	+++	+++	+++

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Experiment 2.—The sodium salts of several organic acids were added in various concentrations to an asparagine medium at pH 6.3. Part of each special medium was kept in the fluid state, and agar in final concentration of 1.5 per cent was added to the other part. The liquid media were distributed in 5 cc. amounts per test tube 25 mm. in diameter and then autoclaved. Each tube was inoculated with 0.5 cc. of the following mixture: 0.05 cc. of 8-day-old culture in Tween albumin medium added to 45 cc. oleic acid–albumin complex and 5 cc. of 50 per cent glucose.

To 50 cc. of each of the sterile agar media (autoclaved and cooled to 55°C.) was added 5.0 cc. of oleic acid–albumin complex and 0.5 cc. of 50 per cent glucose. The mixtures were poured into Petri dishes, and the media allowed to solidify and dry for 24 hours at 37°C. Inoculation was carried out by delivering on the agar surfaces from a tuberculin syringe 0.025 cc. of cultures diluted 10,000-fold (10^{-4}) in 0.1 per cent albumin (17). The agar plates were then sealed with adhesive tape and incubated at 37°C. for 2 weeks. They were then opened and the amount of growth recorded.

TABLE II

Approximate Molar Concentration of Various Organic Acids Required for Complete Inhibition of Growth of Virulent (Human and Bovine) Tubercle Bacilli in Semisynthetic Medium Containing 0.5 Per Cent Oleic Acid–Albumin Complex (pH 6.4)

	Agar medium		Liquid medium	
	H37Rv	Ravenel	H37Rv	Ravenel
Acetic acid	0.0040 M	0.0050 M	0.0050 M	0.0050 M
Propionic acid	0.0030 "	0.0030 "	0.0030 "	0.0060 "
Butyric "	0.0015 "	0.0030 "	0.0020 "	0.0060 "
n Caproic "	0.0002 "	0.0004 "	0.0003 "	0.0005 "
n Caprylic "	0.0003 "	0.0004 "	0.0002 "	0.0004 "
n-Capric "	0.0001 "	0.0002 "	0.0001 "	0.0002 "
Lauric "	0.0002 "	0.0002 "	0.0003"	0.0003 "
Palmitic "	0.0005 "	0.0004 "	0.0010 "	0.0010 "
Stearic "	0.0008 "	0.0006 "	0.0010 "	0.0010 "
Oleic "	0.0010 "	0.0006 "	0.0010 "	0.0010 "
Lactic "	0.0080 "	0.0050 "	0.0100 "	0.0040 "
Pyruvic "	No inhibition at 0.05 M			
Ketoglutaric acid				
Glutamic "				
Succinic "				
Fumaric "				

As seen in Table II, there was complete inhibition of growth with the high concentrations of most of the organic acids. In the liquid media that had received the lower concentrations, the growth consisted of clumps fewer in number and smaller than in the control tubes. On agar media, partial inhibition of growth manifested itself by a decrease in the number and size of the colonies. As no convenient way has been found to record these quantitative differences, only the concentrations of organic acids which caused complete inhibition of growth are recorded in Table II; partial inhibition was readily detectable with

concentrations four, or even eight, times smaller than those required for complete inhibition.

It is clear that inhibition of growth was obtained as readily on solid media as in liquid media, and that the comparative pattern of activity of the different organic acids was essentially the same under both conditions. In fact, whereas the results in liquid media have often shown, from one experiment to the other, inconsistencies still inadequately explained, the experiments carried out on agar surfaces have always proven reproducible.

The data in Table II have been recalculated in terms of molar concentration of organic acids. They reveal that in the saturated aliphatic series, inhibitory activity at first increased with the length of the carbon chain to reach a maximum (in terms of minimum effective molar concentration) with capric acid. From then on inhibitory activity decreased as the molecular weight increased, and even the unsaturated long chain fatty acids proved less active than the C_{10} saturated compound. Lactic acid again proved to exhibit marked inhibitory activity, whereas no inhibition was observed with the highest concentrations used (0.2 per cent) of keto acids (pyruvic and ketoglutaric) and dicarboxylic acids (succinic, fumaric, and glutamic).

The Effect of Composition and Reaction of the Medium on the Inhibitory Activity of Organic Acids.—The results of the two preceding experiments reveal that the growth-inhibitory effect of organic acids takes place whether or not the nutrient solution is enriched with glucose or oleic acid, on agar surfaces as well as in liquid media, in the absence as well as in the presence of a wetting agent promoting submerged growth.

Many other experiments have been performed to determine quantitatively the effect of the different factors of the environment on the inhibition of growth of tubercle bacilli by organic acids. Although no convincing technique has yet been worked out to compare the rate and extent of growth in liquid and on solid media, the trend of the results suggests that the inhibitory activity is slightly greater (a few fold) on the latter than in the former. However, because of the irregularity of the results in liquid media, it does not appear worthwhile to develop this point further at the present time.

The following experiment was designed to test on agar surfaces the effect of other aspects of the composition of the medium on inhibitory activity.

Experiment 3.—Three independent categories of variables were introduced in the test:—

(*a*) Two types of basal media, one containing asparagine and casein hydrolysate as source of nitrogen, the other in which glutamic acid was substituted for asparagine.

(*b*) Three different preparations were compared as source of albumin: fraction V of bovine plasma (0.5 per cent), the oleic acid–albumin complex (0.5 per cent), and whole bovine serum (10 per cent).

(*c*) Three consecutive experiments were done, one at pH 6.1, another at pH 6.5, the third at pH 6.9.

In all cases the sodium salts of organic acids were added in adequate dilutions to the

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TABLE III

Effect of Composition of Medium on Growth-Inhibitory Activity of Organic Acid

Asparagine agar medium		Concentration of organic acid required for 80 per cent inhibition of growth		
pH	Source of albumin (bovine)	Acid	H37Rv	Ravenel
			per cent	*per cent*
6.1	0.5 per cent plasma fraction V	Lactic acid	0.3	0.3
"	" " " " " "	Butyric "	0.02	0.04
"	" " " " " "	Capric "	0.003	0.003
"	" " " oleic acid-albumin complex	Lactic "	0.1	0.03
"	" " " " " " "	Butyric "	0.01	0.01
"	" " " " " " "	Capric "	0.0007	0.0005
"	10.0 " " serum	Lactic "	0.1	0.07
"	" " " "	Butyric "	0.01	0.03
"	" " " "	Capric "	0.001	0.0007
6.5	0.5 per cent plasma fraction V	Lactic "	0.3	0.3
"	" " " " " "	Butyric "	0.03	0.06
"	" " " " " "	Capric "	0.004	0.004
"	" " " oleic acid-albumin complex	Lactic "	0.15	0.05
"	" " " " " " "	Butyric "	0.007	0.007
"	" " " " " " "	Capric "	0.002	0.001
"	10.0 " " serum	Lactic "	0.2	0.2
"	" " " "	Butyric "	0.02	0.02
"	" " " "	Capric "	0.001	0.001
7.0	0.5 per cent plasma fraction V	Lactic "	0.4	0.4
"	" " " " " "	Butyric "	?	?
"	" " " " " "	Capric "	?	?
"	" " " oleic acid-albumin complex	Lactic "	0.4	0.4
"	" " " " " " "	Butyric "	?	?
"	" " " " " " "	Capric "	?	?
"	10.0 " " serum	Lactic "	0.2	0.2
"	" " " "	Butyric "	0.03	0.03
"	" " " "	Capric "	0.003	0.003

different local media prior to autoclaving. After being allowed to dry for 24 hours at 37°C., the agar surfaces were inoculated as described above with 0.025 cc. of 10,000-fold dilutions (10^{-4} in 0.1 per cent albumin) of 8-day-old bacterial cultures in Tween-albumin medium. The Petri dishes were sealed with adhesive tape and incubated at 37°C. The amount of growth was recorded after two weeks' incubation (Table III).

As had been found in Experiment 2, inhibition of growth by organic acids was not an all-or-none phenomenon. Instead, partial inhibition (as expressed by decrease in number and size of colonies) was observed with intermediate concentrations of the acids. In an attempt to facilitate the evaluation of the comparative inhibitory effectiveness of the organic acids tested in the different media, the concentration required to cause approximately 80 per cent reduction in the number of detectable colonies was determined. This was deemed a basis of comparison more valid than complete inhibition since the control media differed in their growth-promoting activity, depending upon their pH and the material used as source of albumin.

The results obtained with the glutamic acid media were so similar to those obtained with the asparagine media that only the latter are recorded (Table III). In general, inhibitory activity was higher the more acid the medium, but the differences were not large. With the four organic acids tested, the inhibitory activity was significantly less (approximately one-third) in the plain albumin media than in media containing the oleic acid-albumin complex or whole serum. This difference was the more striking because growth was less abundant in the control agar plates (without organic acid added) in the first medium than in the last two. No significant difference could be recognized between the media containing oleic acid-albumin complex and those containing whole serum either from the point of view of abundance of growth in the controls, or of the inhibitory activity of the different acids.

Enhancing Effect of Organic Acids on the Growth of Tubercle Bacilli.—It has been reported earlier that certain long chain fatty acids enhance the growth of tubercle bacilli when they are added to the culture medium in admixture with enough serum albumin to neutralize their toxicity (15, 16). The purpose of the following experiment was to determine whether a similar enhancement of growth could be obtained with all organic acids under consideration in the present study.

Experiment 4.—Organic acids were added in various concentrations to asparagine basal medium containing 1.5 per cent agar. The different agar media (at pH 6.4) were autoclaved and 5 cc. of 5 per cent bovine serum albumin was added to 50 cc. of each of them cooled to 50–55°C. They were poured into Petri dishes, allowed to solidify and to dry for 24 hours at 37°C., and then were inoculated by depositing on their surface 0.025 cc. of 10-day-old culture previously diluted to 10^{-3} in 0.1 per cent bovine serum albumin. The inoculated Petri dishes were sealed with adhesive tape and incubated at 37°C. for 3 weeks (Table IV).

As had been found in earlier experiments, the addition to albumin medium of the dicarboxylic and keto acids did not inhibit growth even in the highest concentration used (0.4 per cent), but, on the contrary, resulted in slightly larger colonies (the diameter being increased two- to threefold). In the case of all the aliphatic acids and of lactic acid, growth was increased in the media treated with amounts of organic acids smaller than those required for inhibi-

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tion (Table IV). The range of concentrations capable of enhancing growth was determined in part by the effectiveness of the substances as an inhibitory agent. Thus, capric acid having very high inhibitory activity, enlargement of colonies could be observed only between concentrations of 0.0005 to 0.0015 per cent of this substance. With lignoceric acid, on the other hand, growth stimulation was readily detected between 0.002 and 0.02 per cent. Although the technique of observation did not permit accurate quantitative measurement, it can be stated that enhancement of growth was manifested, not by increased numbers of colonies as compared with those developing on the control media, but by the fact that the colonies became detectable macroscopically a few days earlier, and reached a larger diameter after 3 weeks' incubation. Of the substances tested,

TABLE IV

Enhancement of Growth of H37Rv by Organic Acids

Organic acids added to albumin agar	Maximal concentration giving detectable enlargement of colonies	Effect of higher concentrations
	per cent	
Ketoglutaric acid.....	0.2	No inhibition of growth
Succinic "	0.2	" " " "
Acetic ".....	0.1	Complete inhibition of growth
Propionic ".....	0.05	" " " "
Butyric ".....	0.025	" " " "
Capric ".....	0.0015	" " " "
Lauric ".....	0.002	" " " "
Oleic ".....	0.01	" " " "
Lignoceric ".....	0.02	" " " "
Lactic ".....	0.2	" " " "

lignoceric and oleic acids proved the most effective in stimulating the rate and extent of colonial development.

The Effect of Organic Acids on the Viability of Tubercle Bacilli.—The observations reported so far deal with the effect of organic acids on the proliferation of tubercle bacilli. The following experiment was designed to test the effect of these substances on the viability of the microorganisms.

Experiment 5.—Test tubes 25 mm. in diameter, containing 5 cc. of basal asparagine medium at pH 6.2 with 0.02 per cent Tween 80, received 0.5 cc. of the following respective solutions: sodium lactate (2 per cent), sodium butyrate (0.2 per cent), sodium caprate (0.1 per cent), sodium oleate (0.1 per cent), physiological saline. Each tube was inoculated with 0.5 cc. of culture diluted 100-fold (10^{-2}) in 5 per cent albumin solution in saline. This operation gave culture dilutions of 1,000-fold and a concentration of 0.5 per cent albumin in the medium. The tubes were incubated at 37°C. and observed for the development of growth (Table V). The viability of the bacilli in the different tubes was tested after incubation for 1 hour, 1 day, 2 days, 5 days, 8 days, and 15 days.

The viability test was performed as follows: Tenfold dilutions of the bacterial suspensions were made in physiological saline containing 0.1 per cent albumin. These final dilutions were inoculated on asparagine-oleic acid-albumin agar medium at pH 6.9 by delivering 0.025 cc. on the surface of the agar. The agar plates were sealed with adhesive tape and incubated at 37°C. The number of colonies developing on the plates was counted after 3 weeks' incubation (Table V).

The results presented in Table V show that no visible growth developed in the tubes of Tween-albumin medium that had received sodium lactate, sodium butyrate, sodium caprate, or sodium oleate. Yet, when the contents of these tubes were plated at different intervals of time on a suitable agar medium, the number of colonies obtained gave at first no indication of a decrease in the viability of the cells. A significant decrease in number of colonies was first observed after the inoculum had been in contact with the organic acids for 2 weeks

TABLE V

Survival of Tubercle Bacilli (Bovine Strain Rivenel) in Tween-Albumin Media Containing Inhibitory Concentrations of Organic Acids

Concentration of organic acid in liquid media	Growth in liquid media	Number of viable organisms per cc. of liquid medium after incubation at 37°C. for					
		1 hr.	1 day	2 days	5 days	8 days	15 days
0	++++	10^6	10^6	10^8	10^7	10^8	10^8
Lactic acid 0.5 per cent	—	"	"	10^6	10^6	10^4	10^4
Butyric " 0.05 " "	—	"	"	"	"	10^5	10^4
Capric " 0.005 " "	—	"	"	"	"	10^4	10^3
Oleic " 0.005 " "	—	"	"	"	"	"	10^2

in the Tween-albumin liquid media. It is clear, therefore, that neither lactic, nor butyric, nor capric, nor oleic acid exerted a bactericidal effect on the bacilli and that they only prevented their multiplication.

DISCUSSION

It has been pointed out in earlier studies that many long chain fatty acids inhibit the growth of tubercle bacilli when they are added to synthetic culture media, even in extremely high dilutions, and that this antimicrobial effect can be neutralized in part by adding to the medium sufficient amounts of serum albumin or of sphingomyelin (13-16, 18). The experiments described in the present paper reveal that the shorter chain aliphatic acids, such as acetic, propionic, butyric, caproic, caprylic, and capric acids, also exert a marked inhibitory effect on growth. Indeed, capric acid was the most active of the substances tested, more active in this respect than oleic acid, the toxicity of which has been chiefly emphasized in the past. Inhibition of growth could also be readily demonstrated with low concentrations of lactic acid, but not with the keto and the decarboxylic acids that were tested.

The inhibitory activity of the organic acids appeared to be somewhat higher on albumin-agar media than in the corresponding liquid media. It increased a few fold as the culture medium was made more acidic by addition of HCl. It was significantly higher in media containing whole serum than in those in which serum was replaced by purified albumin, but it was essentially independent of the presence or absence of glucose, and of whether asparagine or glutamic acid was used as the source of nitrogen.

Complete inhibition of growth was found to occur under conditions in which the organic acids did not affect the viability of the inhibited cells. This finding makes it unlikely that the primary cause of bacteriostasis was a disruption of the bacterial membrane resulting from the fact that the substances tested possess in varying degrees surface-depressing properties. It is known, on the other hand, that lactic, butyric, and acetic acid, as well as the long chain fatty acids, can stimulate the oxygen uptake of tubercle bacilli and that this stimulation increases several fold as the test medium is rendered more acidic by addition of HCl (19-22). Interestingly enough, the growth-inhibitory activity of the organic acids followed a parallel course. Moreover, it is known that acetic acid and lactic acid can be used as sources of energy and carbon by tubercle bacilli growing in synthetic media (19, 23). It appears likely, therefore, that, in the present experiments, lactic acid, as well as the short and long chain fatty acids, interfered with the growth of tubercle bacilli by disturbing in some unknown manner the normal course of their metabolism. The slow death of the bacilli resuspended in media containing inhibitory concentrations of organic acids is compatible with this view. Increased respiration, unaccompanied by cellular multiplication, probably caused the exhaustion of essential reserve materials or the accumulation of toxic substances (22) and thus progressively interfered with normal survival.

In adequate concentrations, some of the long chain fatty acids—oleic and lignoceric acid in particular—markedly increased growth in media containing serum albumin. And yet, the inhibitory activity of lactic acid and of the aliphatic acids was increased when the culture media were enriched with the stimulatory concentrations of oleic acid supplied in the form of oleic acid-albumin complex. It is worth noting also that the growth-inhibitory activity of the various organic acids also proved higher in serum media than in plain albumin media. This is of interest since growth in the presence of serum was as good as in oleic acid-albumin media and, therefore, better than in plain albumin media—a fact perhaps due to the known presence in serum of lipids bound to the proteins.

Two hypotheses can be offered to account for the fact that bacteriostatic activity was greatest in the media most satisfactory for growth. On the one hand, it is possible that increased metabolic activity (manifested by better growth) rendered the bacilli more susceptible to the metabolic disturbance

caused by the inhibitory acids. On the other hand, these substances may have displaced the oleic acid, or other lipids, from combination with the albumin or other serum protein with the result that the toxic effect of these lipids was no longer neutralized by the proteins. Available knowledge is not sufficient to permit a choice between these alternative explanations nor to define them better.

Although little is understood of the mechanisms whereby organic acids affect the growth of tubercle bacilli, the known facts make it likely that the findings *in vitro* may be of some significance in analyzing the factors which determine the fate of tubercle bacilli *in vivo*. Some of the organic acids—the keto and dicarboxylic acids—seem to act only as growth promoting agents and never as inhibitors of tubercle bacilli (at least in the range of physiological concentrations). Others—like lactic acid, propionic acid, butyric acid, capric acid, oleic acid, lignoceric acid, etc.—are markedly growth-promoting in low concentrations (particularly near neutrality) and inhibitory in higher concentrations, particularly at more acidic reactions. All these substances are normally produced, either in the course of tissue metabolism, or by autolytic changes following necrosis, and some of them occur *in vivo* in the range of the concentrations which have been found to inhibit bacillary growth in the presence of serum. It seems legitimate, therefore, to postulate that they are not without influence on the evolution of the tuberculous lesion.

As is well known, tubercle bacilli coming into contact with specifically allergic tissues elicit in them a violent inflammatory reaction which may evolve into the caseous state. The site of this reaction becomes acidic, in part because of interference with blood supply, in part perhaps as a result of the accumulation of the large, wandering mononuclear cells which possess a predominantly glycolytic metabolism. It has been reported, furthermore, that fatty acids accumulate in the caseous matter of necrotic areas, probably as a result of lipase activity during autolysis (12, 24, 25). Despite the paucity of convincing quantitative data, one may assume that the concentration of organic acids and the pH prevailing within the inflammatory areas are well within the range of conditions found in the present study to exert a bacteriostatic effect on tubercle bacilli *in vitro*. Compatible with this view is the fact already mentioned that the number of tubercle bacilli detectable in the center of the caseous lesion decreases slowly and continuously as long as the lesion remains closed, whereas the bacilli often start proliferating again when the caseous material softens by repenetration of blood constituents or when the lesion opens into a bronchus or blood vessel. It is true that, as mentioned earlier, increased availability of oxygen may play a part in the release of bacteriostasis. But it is also possible that the ease of exchanges between the lesion and the body fluids may affect in a profound manner the suitability of the environment for bacillary growth by neutralizing the local acidity and decreasing the concentration of growth inhibitors. In other words, it appears worth while to consider that the fate of the

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bacteria present in the lesion is determined, not only by changes in oxygen tension, but also by the local concentration of organic acids and by the efficiency of the acid-base exchanges at the site of the lesion.

Experiments by Lurie are worth recalling in this respect (7). He inserted into the peritoneal cavity of rabbits silk bags containing virulent tubercle bacilli and impregnated with collodion in such a manner as to permit the passage of body fluids, but not of cells. Under these conditions, bacterial multiplication proceeded unchecked in the bags placed in normal animals, whereas little or no growth developed in those in immunized animals. Lurie observed that the reaction of the fluid in the bags in the immunized animals was more acidic than that of the fluid in normal animals, but in no case was the pH low enough (approximately 7.0) to account for bacteriostasis. As phagocytic cells had not penetrated the bags, it appeared that bacteriostasis was caused by some humoral antibody. The results reported in the present paper suggest a different interpretation of Lurie's observation. Lowering of the pH in the immunized allergic animal was probably the result of local accumulation of organic acids caused by the initial inflammatory reaction, and it seems possible that bacterial growth was inhibited, not by the low pH, nor by humoral antibodies, but as a result of the local accumulation of one or several of the organic acids shown in the present study to exhibit bacteriostatic effect on tubercle bacilli.

In order to prevent misunderstanding, it may be necessary to point out here that the presence of organic acids in inflammatory and caseous areas constitutes only one of the several factors which may affect the course of tuberculous infection. Tubercle bacilli are readily phagocytized, and it is probably within the phagocytic cells that the most effective processes of immunity take place. There is, indeed, much evidence that the ability of the infected host to overcome infection ultimately depends upon the power of its monocytes to kill the bacilli that they have engulfed.

SUMMARY

The growth of tubercle bacilli *in vitro* was inhibited by the addition of the sodium salts of very low concentrations of certain organic acids to a variety of liquid and agar culture media containing whole serum or serum albumin.

Capric acid was the most active of the compounds tested, but inhibition of growth occurred also with the shorter aliphatic acids. Lactic acid was also growth-inhibitory, whereas the keto and dicarboxylic acids tested were inactive in this respect.

The inhibitory activity of the aliphatic acids and of lactic acid increased as the pH of the medium was lowered by addition of HCl. It was greater in media enriched with serum or with oleic acid-albumin complex, but was otherwise fairly independent of the composition of the medium.

The inhibitory effect appears to be bacteriostatic rather than bactericidal

and to depend upon a disturbance of the normal metabolic processes of the bacilli.

Some of the long chain fatty acids caused a marked enhancement of growth when used in low concentrations and in admixture with enough serum albumin to overcome their toxicity.

The significance of these findings is discussed with reference to the survival and multiplication of tubercle bacilli *in vivo* within inflammatory and caseous areas, which are known to be often acidic and to contain high concentrations of organic acids.

BIBLIOGRAPHY

1. Rich, A. R., The Pathogenesis of Tuberculosis, Springfield, Illinois, Charles C. Thomas, 1944.
2. Pinner, M., Pulmonary Tuberculosis in the Adult, Springfield, Illinois, Charles C. Thomas, 1945.
3. Canetti, G., Le bacille de Koch dans la lésion tuberculeuse du poumon, Paris, Editions Médicales Flammarion, 1946.
4. Vorwald, A. J., *Am. Rev. Tuberc.*, 1937, 35, 260.
5. Dock, W., and Harrison, T. R., *Am. Rev. Tuberc.*, 1925, **10**, 534.
6. Rich, A. R., and Follis, R. H., Jr., *Bull. Johns Hopkins Hosp.*, 1942, **71**, 345.
7. Lurie, M. B., *J. Exp. Med.*, 1939, **69**, 555.
8. Barnett, G. D., and McKenney, A. C., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1926, **23**, 505.
9. Jervell, O., *Acta med. Scand.*, suppl., 1928, **24**, 1.
10. Kempner, W., and Peschel, E., *Z. klin. Med.*, 1930, **114**, 439.
11. Wells, H. G., and Long, E. R., The Chemistry of Tuberculosis, Baltimore, The Williams & Wilkins Co., 2nd edition, 1932, 180.
12. Menkin, V., Dynamics of Inflammation, New York, MacMillan Company, 1940.
13. Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.
14. Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, 1947, **56**, 334.
15. Dubos, R. J., *J. Exp. Med.*, 1947, **85**, 9.
16. Dubos, R. J., *J. Exp. Med.*, 1948, **88**, 73.
17. Fenner, F., Martin, S. P., and Pierce, C. H., *New York Acad. Sc.*, 1949, **52**, 751.
18. Drea, W. F., *J. Bact.*, 1942, **44**, 149.
19. Loebel, R. O., Shorr, E., and Richardson, H. B., *J. Bact.*, 1933, **26**, 139.
20. Bloch, H., and Suter, W. E., *Schweiz. Z. Path. u. Bakt.*, 1946, **9**, 434.
21. Geronimus, L. H., Ph.D. thesis, Columbus, Ohio State University, 1949.
22. Geronimus, L. H., data to be published, 1950.
23. Schaefer, W. B., *Ann. Inst. Pasteur*, 1948, **74**, 458.
24. Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, **19**, 239, 251, 459.
25. Wells, H. G., and Long, E. R., The Chemistry of Tuberculosis, Baltimore, The Williams & Wilkins Co., 2nd edition, 1932, 169, 181.

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EFFECT OF KETONE BODIES AND OTHER METABOLITES ON THE SURVIVAL AND MULTIPLICATION OF STAPHYLOCOCCI AND TUBERCLE BACILLI

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Uncontrolled diabetes, starvation, as well as other conditions resulting in ketosis, are commonly associated with abnormally high susceptibility to various infections, in particular to those caused by staphylococci and tubercle bacilli. The enhancing effect of hyperglycemia on bacterial multiplication, the decreased activity of the phagocytes (claimed to be related to their high glycogen content), some ill defined defect in antibody production, have all been postulated to play some part in the low resistance to infection of the patient with ketosis. These explanations, however, do not appear to be supported by convincing observations or experiments, and they fail, furthermore, to account for many facts of the infectious process.

The present investigation was stimulated by another working hypothesis, namely that infection during ketosis is facilitated by the additive effect of two independent factors; on the one hand, the presence of ketone bodies in abnormally high concentrations; on the other hand, the decreased production of lactic acid at the site of inflammation. Some experiments dealing with the effect of various metabolites on the survival and multiplication of microorganisms *in vitro* will be presented in support of this hypothesis. The design of these experiments was based on the following considerations.

Rationale of the Work

Lactic acid is known to accumulate in inflammatory areas and to be produced by normal leucocytes (1). Experiments in our laboratory have revealed, furthermore, that lactic acid can exert *in vitro* a bacteriostatic and bactericidal effect on tubercle bacilli, particularly in slightly acidic media with reduced oxygen supply. In contrast, it was found that keto acids have a much lower antibacterial activity under the same conditions (2). These observations suggested the possibility that a disturbance in the lactic acid ketone bodies ratio might create a biochemical environment favorable to infectious agents.

Two reasons made it appear advisable to use acid media to test the validity of this hypothesis: —

(*a*) The intracellular environment of leucocytes has long been known to become very acid immediately following engulfment of a variety of inanimate and living ob-

jects; thus, phagocytic experiments with dyes have revealed that the pH within the leucocytic vacuole may go down to 4.0 or even lower (3). As staphylococci and tubercle bacilli are rapidly phagocytized *in vivo*, it is likely that they are exposed to acid reactions for some time during the intracellular phase of the infectious process.

(*b*) Although no information has been published describing the precise physicochemical environment of inflammatory areas, and in particular their pH, there is much suggestive evidence nevertheless, that the extracellular environment of these areas is also acid, probably as a result of the glycolytic activities of inflammatory cells.

In order to approximate the conditions encountered by microbial agents *in vivo*, experiments were conducted in media consisting of balanced ionic solutions (of the type used in tissue culture), to which serum albumin was added in final concentration of 0.5 per cent and which had been adjusted to acid reactions ranging from neutrality to pH 4.0.

The results obtained in the present study demonstrate that staphylococci and tubercle bacilli die very rapidly at acid reactions in media containing lactic acid in a concentration of 0.01 M (or smaller); acetic, propionic, and butyric acids exert a similar, but less pronounced bactericidal effect. Under the same conditions, the microorganisms survive longer in the presence of polybasic acids (succinic, fumaric, and citric) and of glutamic acid, and more particularly if ketone bodies such as dihydroxyacetone, pyruvic acid, β-hydroxybutyric acid, α-ketoglutaric acid, and oxalacetic acid are added to the suspension fluid. Indeed, ketone bodies can neutralize in part the antimicrobial effect of lactic acid and furthermore, their addition to culture media permits microbial growth at acid reactions (approximately pH 5.3).

Extensive experiments *in vitro* with eight strains of staphylococci and six strains of tubercle bacilli (human and bovine), confirmed by preliminary tests with other microbial species, have thus brought out the fact that lactic acid antagonizes, and ketone bodies favor, the survival and multiplication of certain pathogenic microorganisms in certain acid environments. These opposite effects of the two groups of metabolites suggest that the metabolic disturbances of ketosis may bring about increased susceptibility to infection through two distinct, but perhaps related mechanisms: on the one hand, by depressing the antimicrobial effect of lactic acid at the site of inflammation; on the other, by providing to the infectious agents ketone bodies which act as growth-enhancing factors.

EXPERIMENTAL

Cultures.—The six strains of mammalian tubercle bacilli used in this study have been described in preceding publications under the labels MV, BCG-P, BCG-T, H37Rv, K1Rv, and H37Ra (6). Stock cultures were incubated for 8 to 12 days at 37°C. in the tween-albumin medium commonly in use in our laboratory (5).

Of the eight cultures of staphylococcus that were tested, four were pigmented (orange or lemon yellow) and coagulase-positive, the other four produced white colonies and were coagulase-negative. All were maintained in meat infusion-peptone broth.

In general, it appeared that the avirulent cultures (of tubercle bacilli, and particularly of staphylococci) were more susceptible to the lethal effects of acid reactions than were the virulent cultures. Nevertheless, the general pattern of behavior toward the various metabolites tested was remarkably similar for all cultures. For the sake of brevity, actual results will be presented for only two cultures, namely, "BCG-P" (an attenuated strain of *Mycobacterium tuberculosis bovis*) and "Smith," a pigmented coagulase-positive strain of *Micrococcus aureus*, originally isolated from a patient with osteomyelitis.

Materials.—Most of the substances tested were obtained from commercial sources: Acetic acid (Mallinckrodt Chemical Co., St. Louis, Missouri); butyric and propionic acids (Amend Drug and Chemical Co., Inc., New York); citric and lactic acids (Merck and Co., Inc., Rahway, New Jersey); fumaric acid (Chas. Pfizer and Co., Brooklyn, New York); pyruvic, β-hydroxybutyric, and α-ketoglutaric acids (The Matheson Co., Inc., East Rutherford, New Jersey); glutamic acid (Eastman Kodak Co., Rochester, New York); oxalacetic acid (Krishell Laboratory, Inc., Portland, Oregon).

Confirmatory experiments were carried out also with purified samples of the three following substances: L+ lactic acid (obtained from Dr. Thompson of Ohio State University through the courtesy of Dr. L. H. Geronimus); pyruvic acid and dihydroxyacetone recrystallized and kindly supplied by Dr. R. D. Hotchkiss).

Stock solutions of the different substances were made in distilled water, brought to the desired pH with NaOH, made up to 0.25 M concentration, and sterilized. The solutions of acetate, propionate, butyrate, succinate, fumarate, citrate, glutamate, and lactate were sterilized by autoclaving at 15 pounds' pressure for 10 minutes. On account of their low stability, the solutions of pyruvate, β-hydroxybutyrate, α-ketoglutarate, oxalacetate, and dihydroxyacetone, were prepared immediately before use and were sterilized not by heating, but by filtration through porcelain (Selas) candles. In a few experiments even filtration was omitted in order to minimize any chance of alteration of these substances. The samples were rapidly weighed, dissolved in sterile water, and the reaction of the solution was adjusted by adding to it the required amount of NaOH in the presence of a pH indicator.

Except as indicated in the text, the experiments were conducted in a balanced ionic medium of the following composition:—

NaCl	13.6 gm.
KCl	0.8 "
$MgSO_4 \cdot 7H_2O$	0.2 "
$NaH_2PO_4 \cdot H_2O$	0.29 "
$CaCl_2$	1 drop, 10 per cent
Water	100.0 ml.

The medium was prepared by adding 5 ml. of this stock solution to 95 ml. of distilled water, and adjusting the pH to the desired levels.

Serum albumin (bovine plasma fraction V obtained from the Armour laboratory, Chicago), was added in the form of a 5 per cent solution in saline adjusted to the proper pH and sterilized by filtration through porcelain candles (Selas).

Performance of Tests.—All components of the test medium were adjusted at the proper pH and mixed aseptically. The bacterial suspensions were diluted in 5 per cent albumin (at the proper pH) and added in one-tenth volume to the mixture (giving a final concentration of 0.5 per cent albumin). The final pH was determined by testing an aliquot portion of the mixture with a Beckman glass electrode pH meter. In many experiments, the pH of the mixture was determined again at the end of the incubation period. This was particularly necessary in the case of mixtures containing ketone bodies, since these substances are unstable and undergo alteration rapidly in aqueous medium at 37°C. By using low concentrations of the

ketone bodies in buffered media, it was possible to arrange that the reaction did not change by more than 0.1 pH unit in the course of short term experiments. The only exception was that of oxalacetic acid. This substance decomposed so rapidly under the conditions of the test that only qualitative observations could be made; for this reason, results obtained with oxalacetic acid are not included in the tables.

The time of survival and extent of multiplication of the microorganisms were determined by plating dilutions of the test bacterial suspensions after various incubation periods. Meat infusion-peptone agar was used for staphylococci, and asparagine-albumin agar (5) for tubercle bacilli. The dilutions of staphylococci were made in meat infusion-peptone broth and one loopful of each dilution was streaked on the agar surface. The dilutions of tubercle bacilli were made in albumin solution (without tween) and 1 drop (of approximately 0.025 ml. volume) was deposited from a Pasteur capillary pipette on the agar surface. All dilutions were selected to give well isolated colonies that could be counted (after 24 to 48 hours' incubation for staphylococci, after 2 to 3 weeks' incubation for tubercle bacilli). The numbers of viable units entered in the tables refer to the number of colonies recovered per milliliter of bacterial suspension in the test medium, determined by calculation from the number of colonies actually yielded on agar plates by each dilution.

RESULTS

Viability of Staphylococci and Tubercle Bacilli at pH 4.0 and pH 5.5

To a number of tubes there were added aseptically 2.0 ml. of the balanced mineral solution and 0.2 ml. of 0.25 M (for tubercle bacilli) or 0.1 M (for staphylococci) solutions of the various substances listed in Tables I and II. Two media were used in each case, one at pH 4.0, the other at pH 5.5. The tubes were inoculated with 0.2 ml. of culture of BCG-P (10 days old) or of staphylococcus Smith (2 days old) diluted 1,000-fold in 5 per cent albumin (at pH 4.0 and at pH 5.5). In order to minimize evaporation, the cotton plugs of the tubes containing tubercle bacilli were sealed with paraffin. All bacterial suspensions were placed at 37°C. and the numbers of living organisms determined by plating on agar after 1, 2, 3, 5, 24, and 48 hours in the case of staphylococci, and 1, 2, 3, 5, 7, 10, and 14 days in the case of tubercle bacilli. The numbers of viable units (calculated per milliliter of bacterial suspension) are indicated in Tables I and II.

As was to be expected, the tubercle bacilli survived much longer than the staphylococci and the microorganisms of both groups died more rapidly at pH 4.0 than at pH 5.5. Except for a few minor exceptions (particularly with reference to their behavior toward glutamic acid) both groups of microorganisms exhibited the same pattern of susceptibility to the various substances tested. Survival at both reactions was much shorter in the solutions containing lactic acid than in the solutions with no organic acid added. Acetic, butyric, and propionic also exerted a bactericidal effect, but less rapid than that of lactic acid. Succinic, fumaric, and citric acid proved less toxic than lactic and the aliphatic acids. Glutamic acid was remarkably innocuous for tubercle bacilli, but somewhat less for staphylococci. Survival was longest in tubes containing dihydroxyacetone, pyruvic, β-hydroxybutyric, α-ketoglutaric, and oxalacetic acids. Unfortunately, the pH of the solutions of oxalacetic acid increased in the course of incubation and for this reason it was not considered justifiable to include in the tables the results obtained with this substance.

TABLE I

Effect of Various Metabolites on Viability of Staphylococci at Acid Reactions

Substance added to suspension of staphylococci (Final concentration 0.01 M)	No. of viable staphylococci* after exposure at							
	pH 4.0 for (*hrs.*)				pH 5.5 for (*hrs.*)			
	1	2	3	5	2	5	24	48
Control	3×10^5	1×10^5	6×10^4	1×10^4	3×10^5			2×10^5
Acetic acid	2×10^5	3×10^4	0	0	1×10^5	1×10^4	0	0
Propionic "	2×10^5	3×10^4	1×10^4	0	2×10^5	1×10^5	1×10^4	
Butyric "	3×10^5	8×10^4	3×10^4	0	3×10^5	1×10^5	2×10^4	
Succinic "	3×10^5	2×10^5	1×10^5	0	3×10^5	2×10^5	5×10^4	
Fumaric "	3×10^5	2×10^5	1×10^5	0	3×10^5	3×10^4	3×10^4	
Citric "	3×10^5	2×10^4	6×10^4	0	3×10^5	2×10^5	4×10^4	
Lactic "	1×10^5	2×10^4	0	0	5×10^4	0	0	
Pyruvic "	3×10^5	3×10^5	1×10^5	6×10^4	3×10^5	4×10^4	1×10^5	5×10^4
β-Hydroxybutyric "	3×10^5	2×10^5	9×10^4	1×10^4	3×10^5	2×10^5	2×10^5	1×10^5
α-Ketoglutaric "	3×10^5	3×10^5	2×10^5	1×10^5	3×10^5	3×10^4	4×10^5	7×10^5
Glutamic "					3×10^5	2×10^4	2×10^4	9×10^3
Dihydroxyacetone	3×10^5	3×10^5	3×10^5	2×10^5	3×10^5	4×10^5	4×10^5	6×10^5

* Determined by duplicate plating on meat infusion-peptone agar.

TABLE II

Effect of Various Metabolites on Viability of Tubercle Bacilli at Acid Reactions

Substance added to suspension of bacilli (Final concentration 0.025 M)	No. of viable bacilli* after exposure at									
	pH 4.0 for (*days*)					pH 5.5 for (*days*)				
	1	2	3	6	8	3	5	7	10	14
Control	10^5	10^5	10^5	10^3	0	10^5	10^5	10^5	10^5	10^4
Acetic acid	10^4	10^3	0	0	0	10^2	0	0	0	0
Propionic "	10^4	10^3	0	0	0	10^5	10^5	10^4	10^3	0
Butyric "	10^4	10^5	0	0	0	10^5	10^5	10^4	10^4	0
Succinic "	10^5	10^5	10^4	0	0	10^5	10^5	10^5	10^4	10^2
Fumaric "	10^5	10^5	10^4	0	0	10^5	10^5	10^5	10^5	10^7
Citric "	10^5	10^5	10^5	0	0	10^5	10^5	10^5	10^5	10^4
Lactic "	10^4	0	0	0	0	0	0	0	0	0
Pyruvic "	10^5	10^5	10^5	10^4	10^2	10^5	10^5	10^5	10^4	10^3
β-Hydroxybutyric "	10^5	10^5	10^3	0	0	10^5	10^5	10^5	10^5	10^3
α-Ketoglutaric "	10^5	10^5	10^5	10^2	0	10^5	10^5	10^5	10^5	10^5
Glutamic "	10^5	10^5	10^5	0	0	10^5	10^5	10^5	10^5	10^5
Dihydroxyacetone	10^5	10^5	10^5	0	0	10^5	10^5	10^5		
Glycine	10^5	10^5	10^5	10^2	0	10^5	10^5	10^5	10^4	10^3
Alanine	10^5	10^5	10^4	0	0	10^5	10^5	10^4	10^3	10^2

* Determined by duplicate plating on albumin-asparagine agar. Calculated to the nearest exponential value of 10.

TABLE III

Effect of Various Metabolites on Toxicity of Acetic and Lactic Acids for Staphylococci at Acid Reactions

Substances added to suspension of staphylococci (Final concentration 0.02 M)	No. of viable staphylococci* after exposure at								
	pH 4 for (*hrs.*)				pH 5.5 for (*hrs.*)				
	2	3	4	5	3	5	8	24	48
Acetic acid	1×10^5	4×10^4	0	0	3×10^4	0	0	0	0
" " + pyruvic acid	3×10^5	1×10^5	7×10^4	0	2×10^5	3×10^5	0	0	0
" " + α-ketoglutaric "					3×10^5	2×10^5	9×10^4	4×10^4	0
" " + β-hydroxybutyric "	8×10^5	3×10^5	0	0	2×10^5	8×10^5	2×10^5	0	0
" " + dihydroxyacetone	2×10^5	3×10^4	1×10^4		7×10^4	1×10^5	0	0	0
Lactic acid	2×10^5	0	0	0	4×10^5	0	0	0	0
" " + pyruvic acid	7×10^5	2×10^5	0	0	2×10^5	3×10^5	0	0	0
" " + α-ketoglutaric "					3×10^5	1×10^5	7×10^5	2×10^5	1×10^4
" " + β-hydroxybutyric "	2×10^5	5×10^4	0	0	3×10^5	2×10^5	3×10^5	0	0
" " + dihydroxyacetone	9×10^5	3×10^5	0	0	1×10^5	4×10^5	0	0	0

* Determined by duplicate plating on meat infusion–peptone agar.

TABLE IV

Effect of Various Metabolites on Toxicity of Acetic and Lactic Acids for Tubercle Bacilli at Acid Reactions

Substances added to suspension of bacilli (Final concentration 0.025 M)	No. of viable bacilli* after exposure at							
	pH 4 for (*days*)				pH 5.5 for (*days*)			
	1	2	3	6	3	5	7	10
Acetic acid	9×10^6	7×10^5	0	0	8×10^5	0	0	0
" " + pyruvic acid	2×10^6	5×10^5	0	0	9×10^5	3×10^5	0	0
" " + α-ketoglutaric "	3×10^6	1×10^6	4×10^4	2×10^5	3×10^6	2×10^5	7×10^4	1×10^4
" " + β-hydroxybutyric "	2×10^6	3×10^4	0	0	1×10^6	1×10^6	0	0
" " + glutamic "					2×10^6	2×10^5	4×10^5	7×10^5
Lactic acid	4×10^6	0	0	0	2×10^6	0	0	0
" " + pyruvic acid	1×10^5	6×10^5	0	0	0	0	0	0
" " + α-ketoglutaric "	3×10^5	4×10^5	0	0	2×10^5	1×10^5	0	0
" " + β-hydroxybutyric "	8×10^4	4×10^3	0	0	5×10^3	2×10^3	0	0
" " + glutamic "					1×10^5	2×10^5	4×10^5	9×10^5
" " + dihydroxyacetone					2×10^6	4×10^5	1×10^5	3×10^6

* Determined by quadruplicate plating on asparagine–albumin agar.

Partial Neutralization of the Antibacterial Effect of Lactic and Acetic Acids by Various Metabolites

Tubes containing 2 ml. of the balanced ionic solution with 0.025 M of either acetic or lactic acid, at pH 4.0 and pH 5.5, received in addition 0.2 ml. of physiological saline, or of 0.25 M solutions of dihydroxyacetone, or pyruvic, β-hydroxybutyric, α-ketoglutaric, or oxalacetic acid. The tubes were then inoculated with 0.2 ml. of suspensions of staphylococci, or of tubercle bacilli, that had been diluted 1000-fold in 5 per cent albumin at pH 4.0 or pH 5.5.

The viability of the organisms was tested after various incubation periods, as described in the preceding experiment. The numbers of viable units (calculated per milliliter of suspension) are given in Tables III and IV.

The results presented in Tables III and IV reveal that the survival time of staphylococci and tubercle bacilli in the presence of acetate and lactate at acid reaction was slightly increased by addition of ketone bodies to the test systems. Of the substances listed in the tables, α-ketoglutaric acid proved to be most effective in its protective effect. Even more satisfactory results were obtained with oxalacetic acid. Unfortunately, as already observed in the case of the preceding experiment, the pH of the media containing this substance increased during incubation, thus preventing adequate quantitative comparisons.

TABLE V

Effect of Age of Culture on Susceptibility of Staphylococci to Lactic Acid

Age of culture	Final concentration of lactic acid	No. of viable staphylococci* after exposure at pH 4.0 for (*hrs.*)		
		4	10	16
6 hrs.	0	2×10^4	3×10^3	0
"	0.01 M	7×10^2	8×10^1	0
"	0.025 M	5×10^2	8×10^1	0
8 days	0	1×10^5	8×10^4	6×10^3
"	0.01 M	7×10^4	5×10^3	2×10^2
"	0.025 M	5×10^4	6×10^3	9×10^1

* Determined by duplicate plating on meat infusion–peptone agar.

Effect of Age of the Culture on the Survival of Staphylococci at Acid Reactions

In the course of the many tests that were performed in order to determine the effect of various metabolites on the viability of staphylococci at acid reactions, it was observed that—with any given strain—the rate of death in the tubes having received the test substances, as well as in control tubes, differed markedly from one experiment to the other. As every effort had been made to carry out the tests under identical environmental conditions, it appeared likely that the origin of the differences in results resided in the staphylococci themselves. Among the many factors that might influence the susceptibility of staphylococci to the bactericidal effect of acid media, only one has been studied so far, namely the age of the culture.

Two cultures of the "Smith" strain of staphylococcus were compared, one 6 hours old still in the logarithmic period of growth, the other 8 days old. The latter was stored at night in the refrigerator throughout the week, but kept at room temperature for several hours every day during that period. The viability of the two cultures in the presence and absence of lactic acid was determined by techniques similar to those used in the two preceding experiments.

The results presented in Table V make clear that the staphylococci of the older culture (8 days) survived much longer than did the organisms in the logarithmic phase of growth whether or not lactic acid was present in the suspension fluid at pH 4.0. In other tests not reported here, it was found that the older staphylococci did survive for several weeks at pH 5.5, whereas the young cells died within a very few days under the same conditions. Unfortunately, technical problems have made it difficult so far to determine the effect of age of the culture on the resistance of tubercle bacilli to various metabolites in acid media.

Effect of Various Metabolites on the Multiplication of Staphylococci and Tubercle Bacilli at Acid Reactions

The results presented in Tables I and II suggest that, at pH 5.5, there took place some multiplication of the staphylococci and tubercle bacilli resuspended in solutions containing ketone bodies or glutamic acid. Experiments were, therefore, instituted to ascertain this fact in a more convincing manner.

A basal medium of the following composition was prepared:

0.5 M Na_2HPO_4	50.0 ml.
0.5 M KH_2PO_4	5.0 "
0.05 M $MgSO_4$	6.5 "
0.05 M $CaCl_2$	6.5 "
3 per cent yeast extract	6.5 "
H_2O to	650.0 "

Aliquots of this medium were adjusted to reactions ranging from pH 7.0 to pH 4.5. After sterilization by autoclaving, serum albumin (at pH 5.5) was added aseptically in a final concentration of 0.5 per cent. The albumin media were then distributed in 3 ml. amounts in a number of tubes to each of which was added 0.3 ml. of oxalacetic acid or of solutions of the various metabolites listed in Tables VI and VII. These had been adjusted to reactions ranging from pH 4.5 to 7.0 before addition to the media of the corresponding pH.

The tubes were inoculated with 0.3 ml. of staphylococcus cultures (2 days old) diluted 100-fold in 2 per cent glucose solution in water. In a similar experiment the tubes of a duplicate set were inoculated with 0.3 ml. of culture of tubercle bacilli diluted 10-fold in glucose solution. The cultures were incubated at 37°C. and the extent of growth was estimated after 12 hours' incubation for the staphylococci, and 4 days' for the tubercle bacilli.

The pH of each of the media was determined (by use of the glass electrode) on an aliquot before incubation, and again at the end of the experiment. Only data referring to media, the reaction of which did not change by more than 0.2 pH unit, are included in Tables VI and VII. Because of the large numbers of variables involved, it was not practically possible to perform all the tests simultaneously. In consequence, Tables VI and VII present composites of the results of several experiments.

Several conclusions appear obvious from examination of Tables VI and VII. It can be seen that the minimum and optimum pH for growth depended not only upon the species of organism used, but even more upon the composition of the medium. With staphylococci for example, growth took place at pH 5.3 in the pyruvic acid medium, but only at reactions near neutrality in the lactic

and acetic acid media. Similarly, growth of tubercle bacilli was observed at a pH as low as 5.2, in media containing α-ketoglutaric acid, dihydroxyacetone,

TABLE VI

Effect of Various Metabolites on the Multiplication of Staphylococci at Acid Reactions

Substance added to the medium (Final concentration 0.025 M)	Growth* after 18 hrs.' incubation (37°C.) at indicated pH			
	5.3	5.5	5.8	6.9
0	−	++	++++	+++
Acetic acid	−	−	−	+++
Propionic "	−	−	−	
Butyric "	−	−	−	
Lactic "	−	+	++	++++
Fumaric "	−	−	+++	++++
Pyruvic "	++	++++	++++	+++
α-Ketoglutaric "	−	+	+++	++++
β-Hydroxybutyric "	+	++	+++	++
Glutamic "	−	++	++	++++
Dihydroxyacetone		++++		

* Recorded according to an arbitrary scale (visual observation) from − (no growth) to ++++ (maximum growth).

TABLE VII

Effect of Various Metabolites on the Multiplication of Tubercle Bacilli at Acid Reactions

Substance added to the medium (Final concentration 0.025 M)	Growth* after 4 days' incubation (37°C.) at indicated pH					
	4.5	5.2	5.5	5.8	6.4	6.9
0	−	−	++	++	+++	++
Acetic acid	−	−	−	−		+++
Propionic "	−	−	−	−		+++
Butyric "	−	−	−	−		+++
Lactic "	−	−	−	−	+++	++++
Fumaric "	−	−	++++	++++	++++	++++
Pyruvic "	−	−	−	++	++++	+++
β-Hydroxybutyric "	−	−	−	+++	++++	++
α-Ketoglutaric "	−	++	++++	++++		++
Glutamic "	−	+++	++++	++++	++++	++++
Dihydroxyacetone	−	++	+++	+++	+	−

* Recorded according to an arbitrary scale (visual observation) from − (no growth) to ++++ (maximum growth).

and glutamic acid but only at reactions nearer to neutrality in the presence of acetic, propionic, butyric, and lactic acids. Furthermore, lactic acid which was inhibitory to the growth of both types of microorganisms, at acid reactions, did enhance their growth at neutrality. The opposite situation was observed

with dihydroxyacetone, which enhanced the growth of tubercle bacilli at acid reactions, and inhibited it at reactions higher than pH 6.5. (As dihydroxyacetone is unstable under the conditions of the test, it is possible that this inhibitory effect was due not to dihydroxyacetone itself, but rather to its decomposition products at or near neutrality.)

Thus, growth experiments reveal that lactic acid (and also some short aliphatic acids) inhibit, and ketone bodies stimulate, the multiplication of staphylococci and tubercle bacilli at the acid reactions which can be assumed to prevail in inflammatory areas. These findings in culture media confirm and extend those of the viability tests performed in mineral solutions. Needless to say, there are many complex factors which are bound to alter the play of these biochemical forces *in vivo*. The results of the present study make it appear likely nevertheless that ketosis, as well as other metabolic disturbances, can exert a profound influence on the course of infection, by affecting the survival and multiplication of microorganisms through changes in the biochemical environment—local or general, intracellular or extracellular.

SUMMARY

A study has been made of the fate of staphylococci and tubercle bacilli resuspended in aqueous media at slightly acid reactions. The tests were carried out at several acid reactions in balanced ionic media containing 0.5 per cent serum albumin. These experimental conditions were selected in order to approximate those which are probably encountered by pathogenic agents in inflammatory areas and in the intracellular environment of the leucocytes after phagocytosis.

The viability of the microorganisms at a given pH was markedly influenced by the composition of the medium, being decreased by addition to the latter of lactic, acetic, propionic, and butyric acids, and increased by the addition of certain ketone bodies such as dihydroxyacetone and pyruvic, β-hydroxybutyric, α-ketoglutaric, and oxalacetic acids.

The presence of ketone bodies in the medium afforded to the microorganisms some protection against the bactericidal effect of lactic and acetic acids at acid reactions.

The minimum and the optimum pH for growth were found to be dependent on the composition of the medium. Both were higher in the presence of lactic, acetic, propionic, and butyric acids than in the media without organic acids added. In contrast, the addition of ketone bodies to the medium allowed microbial multiplication even in acid media (approximately at pH 5.3 or even lower).

The fact that lactic acid antagonizes, whereas ketone bodies favor, the survival and multiplication of staphylococci and tubercle bacilli at acid reactions, is discussed in relation to the high susceptibility to infection which is often associated with ketosis of various etiology.

BIBLIOGRAPHY

1. (*a*) Barnett, G. D., and McKenney, A. C., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1926, **23**, 505.
   (*b*) Fleischmann, W., and Kubowitz, F., *Biochem. Z.*, 1927, **181**, 395.
   (*c*) Jervell, O., *Acta med. scand.*, 1928, **24**, suppl., 1.
   (*d*) Kempner, W., *J. Clin. Inv.*, 1939, **18**, 291.
   (*e*) Kempner, W., and Peschel, E., *Z. Klin. Med.*, 1930, **114**, 439.
   (*f*) McLeod, J., and Rhoads, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 268.
   (*g*) Menkin, V., Dynamics of Inflammation, New York, Macmillan Co., 1940.
   (*h*) Stetson, C., *J. Exp. Med.*, 1951, **93**, 489; **94**, 347.
   (*i*) Valentine, W. W., *Blood*, 1951, **6**, 845.
   (*j*) McKinney, G. R., Rundles, R. W., and Green, R., *J. Appl. Physiol.*, 1953, **5**, 7, 335.
2. Dubos, R. J., *J. Exp. Med.*, 1950, **92**, 319; 1953, **97**, 357.
3. (*a*) Rous, P., *J. Exp. Med.*, 1925, **41**, 379, 399, 451.
   (*b*) Pulcher, C., *Boll. soc. ital. biol. sper.*, 1927, **2**, 223, 722.
   (*c*) Ishikawa, A., *Z. Klin. Path. u. Hämatol.*, 1935, **4**, 305; *Nagoya J. Med. Sc.*, 1936–37, **10–11**, 257.
4. Pierce, C. H., Dubos, R. J., and Schaefer, W. B., *J. Exp. Med.*, 1953, **97**, 189.
5. Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, 1947, **56**, 334.

THE EFFECT OF CASTRATION ON THE INCORPORATION OF ACETATE-1-C^{14} INTO CHOLESTEROL IN THE ALBINO RAT[1]

LOUIS-PAUL DUGAL AND GUY SAUCIER[2]

Abstract

In male castrated albino rats the incorporation of C^{14} from acetate-1-C^{14} into the cholesterol of the liver, the adrenals, and the heart is significantly increased as compared to the incorporation found in the same tissues of control animals. The metabolic $C^{14}O_2$ originating from the injected acetate is not affected by castration, nor is the total activity of the urine. There is, incidentally, a significant increase in adrenal weight following castration.

Introduction

The metabolism of cholesterol has regained much interest since the more general use of isotopic techniques (3). Successful studies of cholesterol metabolism may give important leads in such problems as atherosclerosis and hormonal steroidogenesis.

Many studies are at present in progress on cholesterol synthesis (1, 8, 11) and on the effect of lipid feeding and hormonal control of plasma cholesterol in rats, chickens, and rabbits (6, 9). The purpose of the present work was to find out the possible influence of castration on the incorporation of C^{14} from acetate-1-C^{14} into the cholesterol of different tissues of castrated albino rats as compared to normal ones. Landon and Greenberg (7) have reported a decrease in the turnover of cholesterol in castrated rats. In the same paper, they state that: "Liver slices of castrated male rats converted C^{14}-acetate to cholesterol at least as efficiently as the controls", but they give no supporting data.

Methods

Twenty-six male albino rats of an average weight of 175 g. were bilaterally gonadectomized by abdominal approach under light ether anesthesia. They were fed Purina fox chow and water, without added cholesterol. Between 12 to 54 days after castration, they were injected intraperitoneally with 2 ml. sodium acetate-1-C^{14} solution equivalent to 1.7×10^7 c.p.m., then immediately placed in a metabolism cage (5) to collect excreted urine and trap expired $C^{14}O_2$ by means of towers filled with 2 *N* sodium hydroxide.

Four hours after the injection, they were sacrificed by guillotine, and the liver, the adrenals, the heart, and the aorta were removed at once; from these tissues, cholesterol was extracted (14) and plated as digitonide on filter paper, weighed, and placed in the counter. The metabolic $C^{14}O_2$, trapped in sodium hydroxide, was precipitated, after proper dilution, with 1 *M* barium chloride

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TABLE I

EFFECT OF CASTRATION ON C^{14} INCORPORATION IN LIVER CHOLESTEROL

	Liver weight		Cholesterol			
	Abs. (g.)	% body wt.	Total (mg.)	%	Total c.p.m.	c.p.m./mg. cholesterol
Controls (13)	10.2 ± 0.33*	3.29 ± 0.11	15.3 ± 0.53	0.15 ± 0.0067	28167 ± 4013	1888 ± 271
Castrated (13)	8.9 ± 0.24	3.42 ± 0.13	14.7 ± 0.78	0.16 ± 0.010	37065 ± 1969	2763 ± 246
P	<0.01	<0.50	<0.50	<0.50	<0.10	<0.05

*Standard error.
In parentheses, number of animals.

and counted as $BaC^{14}O_3$ (4). The urine samples, after combustion (10, 15), were analyzed for their total activity. All radioactivity measurements were made in a Q-gas counter with window, and corrected for background and self-absorption.

Results and Discussion

Table I shows that the specific activity of the liver cholesterol is significantly greater in the castrated group than in the control, i.e., there were more newly synthesized cholesterol molecules after 4 hours than in the control group. There is also a difference in the total number of counts per minute, which is not significant owing to too great individual variations. The difference in absolute weights of liver, between the two groups, is due to the retarded growth induced by castration; such a difference is abolished when the relative weights (to body weight) are compared.

The adrenals of the castrated group, as can be seen in Table II, are larger than those of the control group, and the difference is statistically significant, whether the comparison is absolute or relative. The same table also shows that castration increases the cholesterol content (not significantly) (13), the total counts, and the specific activity of the adrenals; the increase in total counts is highly significant, whereas the increase in specific activity is not, owing to the large individual variations in both groups.

TABLE II

EFFECT OF CASTRATION ON C^{14} INCORPORATION IN ADRENAL CHOLESTEROL

	Adrenal weight		Cholesterol			
	Abs. (g.)	% body wt. ×10³	Total (mg.)	%	Total c.p.m.	c.p.m./mg. cholesterol
Controls (13)	33.5 ± 1.42*	10.9 ± 0.41	0.41 ± 0.068	1.32 ± 0.20	175 ± 46	464 ± 107
Castrated (13)	39.6 ± 1.77	15.0 ± 0.59	0.67 ± 0.11	1.81 ± 0.31	380 ± 52	691 ± 150
P	<0.02	<0.001	<0.10	<0.20	<0.01	<0.20

*Standard error.
In parentheses, number of animals.

TABLE III

EFFECT OF CASTRATION ON C^{14} INCORPORATION IN HEART CHOLESTEROL

	Heart weight		Cholesterol			c.p.m./mg. cholesterol
	Abs. (g.)	% body wt.	Total (mg.)	%	Total c.p.m.	
Controls (13)	1.04 ± 0.025*	0.337 ± 0.092	1.13 ± 0.07	0.108 ± 0.0065	357 ± 71	308 ± 54
Castrated (13)	1.10 ± 0.059	0.367 ± 0.148	1.10 ± 0.08	0.114 ± 0.0086	494 ± 54	406 ± 54
P	<0.50	<0.90	<0.80	<0.70	<0.20	<0.05

*Standard error.
In parentheses, number of animals.

There were no significant differences in heart weight or heart cholesterol between the two groups (Table III). Here again, a constant higher counting rate was observed in the castrated animals, and the specific activity was significantly higher in the same group.

Table IV shows the results obtained in comparing the activity of expired $C^{14}O_2$ and total activity of excreted urine in both groups; the similarity of the figures obtained points to the metabolic similarity of both groups.

TABLE IV

EFFECT OF CASTRATION ON THE RESPIRATORY $C^{14}O_2$ AND THE URINE C^{14} CONTENT

	$C^{14}O_2$ (c.p.m.×10^7)	Urine total activity (c.p.m.×10^9)
Controls (13)*	1.20	3.69
Castrated (13)	1.26	3.73

*In parentheses, number of animals.

Assuming that a lapse of 4 hours is a significant point in the time curve of endogenous cholesterol synthesis from acetate (2) and that mainly cholesterol is precipitated by digitonin, without contamination from "high counting companions" (12), the differences presented above, which are probably due to orchidectomy, may be of interest. However, since they were obtained at one time interval only, they cannot be interpreted in terms of rate of synthesis nor of turnover rate.

References

1. BLOCH, K. Harvey lectures 1952–53. New York Academy Press. 1954.
2. ECKLES, N. E., TAYLOR, C. B., CAMPBELL, D. J., and GOULD, R. G. J. Lab. Clin. Med. 46, 359 (1955).
3. FREEDMAN, M., BYERS, S. O., and ST. GEORGE, S. Ann. Rev. Biochem. 25, 613 (1956).
4. HENRIQUES, F. R., JR., KISTIAKOWSKY, G. B., MARGUETTI, C., and SCHNEIDER, W. G. Ind. Eng. Chem., Anal Ed. 18, 349 (1946).
5. JACKEL, S. S., MOSBACH, E. H., BURNS, J. J., and KING, C. G. J. Biol. Chem. 186(2), 569 (1950).
6. KATZ, L. N., STAMLER, J., and PICK, R. Symposium on atherosclerosis. Natl. Acad. Sci. N.R.C. 338, 236 (1955).

7. LANDON, E. J. and GREENBERG, D. M. J. Biol. Chem. 209, 493 (1954).
8. LANGDON, R. C. and BLOCH, K. J. J. Biol. Chem. 200, 129 (1953).
9. LIN, T. N., KARVINEN, E., and IVY, A. C. Am. J. Physiol. 183, 86 (1955).
10. LINDENBAUM, A., SCHUBERT, J., and ARMSTRONG, W. D. Anal. Chem. 20, 1120 (1948).
11. ROSENMAN, R. H., FRIEDMAN, M., and BYERS, S. O. Endocrinology, 51, 112 (1952).
12. SCHWENK, E., ALEXANDER, G. S., and FISH, C. A. Arch. Biochem. 58, 37 (1955).
13. SIMONNET, H., MICHEL, E., and SEGAL, V. Compt. rend. soc. biol. 142, 579 (1948).
14. SPERRY, W. N. J. Biol. Chem. 118, 377 (1937).
15. VAN SLYKE, D. D., PLAZIN, J., and WEISIGER, J. R. J. Biol. Chem. 191, 299 (1951).

Viability of Thermophilic Bacteria in the Presence of Varying Concentrations of Acids, Sodium Chloride and Sugars[a]

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A study was made of the influence of acids (acetic, citric and lactic), sodium chloride and sugars (dextrose, sucrose, fructose) on the viability of five representative cultures of thermophilic bacteria.

The order of effectiveness of the acids in terms of germicidal action was found to be citric > acetic > lactic. For the sugars the order was found to be dextrose > fructose > sucrose. Concentrations of sodium chloride above 1.5 per cent proved germicidal while concentrations below 1.0 per cent accelerated growth in some cases.

In all cases normal growth was observed to be very rapid, the maximum cell count being attained after about eight to sixteen hours. This exceptionally high growth rate was followed by a pronounced and rapid death rate, sterility of some cultures ensuing after 48-96 hours.

Important as thermophilic bacteria are in the food industry, especially in the spoilage of canned foods, the literature reveals only a paucity of information on their classification and physiological characteristics. Schmidt *(21)* significantly remarked "The group of organisms known as obligate thermophiles has been completely neglected."

Thermophilic spoilage has for years been a vexing problem to food canners, food technologists and food bacteriologists. The ubiquitous nature of the thermophilic bacteria and the extraordinarily high heat resistance of their spores greatly increase the probability of spoilage in industry. They may occur in food ingredients such as sugar and starch. The spoilage of sugar in storage and during shipment has been attributed to their action. Several cases of wholesale loss of inadequately processed canned foods due to thermophilic spoilage have been reported. Beans, peas, corn and non-acid foods in general, as well as tomatoes, are very susceptible. In all such cases the flat sour obligate thermophiles, in the sense of the definition of Cameron and Esty *(5)*, are of particular importance.

The retreat of the Allied armies in the North African campaign during World War II was ascribed by some to be due in part to a shortage of rations incurred as a result of thermophilic spoilage of canned foods stored in the warm African desert. Spoilage of canned foods due to the same cause occurred in the Pacific. With the current strategic importance of the Pacific theatre and with large shipments of edible materials pouring into these localities, thermophilic spoilage is again a problem of impressive stature. Further close study of the behavior of these organisms may therefore be profitable.

Acids, salt and sugar occurring either naturally or added in varying proportions play an important role in the vast modern enterprise of food preservation; their preservative action was recognized even by primitive man. The hydrogen-ion concentration of food influences its deterioration by microorganisms. Concentrated solutions of salt and sugars are used to preserve certain foods by repressing development of microorganisms; meat, pickles, cabbage, onions, peppers, cauliflower and other vegetables being preserved by salt and many fruits by sugar sirups.

The influence of acids, salt and sugars on thermophilic bacteria has not been extensively studied. As a result, opinions differ widely as to the kind and quantity of any of these ingredients to be used for effective but, at the same time, economical preservation. The general objective of these studies was to ascertain the trend of growth of thermophilic bacteria in a specific medium which had been adjusted to different pH levels with different acids or to which increasing concentrations of salt or sugar had been added. It was decided that the role which acid, salt and sugar play in the preservation of foods should be determined—specifically, (a) The order of effectiveness, bacteriostatically and bactericidally, of certain acids, salt and sugars against thermophilic bacteria. The acids were used to adjust the medium to the same pH level while the salt and sugars were added in increasing concentrations. (b) The minimum, or probable, time for which the medium so treated and inoculated with thermophilic cultures would have to be incubated in order to reduce the bacterial population. (c) The relative rates of death of thermophilic bacteria isolated from various sources when incubated for varying periods in a medium so treated.

LITERATURE REVIEW

A very lucid review of the literature on the nature of thermophiles is given by Gaudhran *(8)*, but a brief resumé will be useful as background for the present study.

Thermophilic bacteria have been variously characterized. They may be simply defined as unicellular spore-forming microorganisms depending for their existence on animal or plant tissues and which grow at relatively high temperatures. Crickett *(17)* after sifting the various definitions accepted the one given by Cameron and Esty *(5)* who divided them into obligate and facultative thermophilic bacteria. Insenecki and Solazova *(11)* have divided them, on a temperature basis, into stenothermal and euritherual thermophiles, while Bergey *(1)* classifies them as true and facultative thermophiles.

Gaudhran *(8)* points out that the multiple connotations of the terms used to describe thermophiles have led to much misinterpretation. He thinks that bacteria with any optimum growth temperature of between 50° C. and 60° C. may be called thermophiles and suggests that, if necessary, the terms of Insenecki and Solazova *(11)* should be used for defining the magnitude of the temperature range for growth.

The effect of acids. Erickson and Fabian *(8)* showed that thermophiles are more susceptible to increasing concentrations of acids than mesophiles. Anderson and coworkers *(1)* demonstrated that lactic, acetic, and citric acids in increasing concen-

[a] Journal Article No. 1428.

trations reduced the thermal destruction rates of *Bacillus thermoacidurans*, the relative order of efficiency being lactic > citric > acetic. Pederson and Becker *(16)* concluded that there seems to be a significant difference between the minimum pH for the growth of vegetative cells and the minimum pH for the germination of spores of *B. thermoacidurans*: vegetative cells initiated growth in broth of pH as low as 4.38, while heat-resistant spores were incapable of germination at pH below 5.0. Nunheimer and Fabian *(15)* found that on a pH basis, the order of effectiveness for the germicidal action of the acids against strains of food poisoning staphylococci was acetic > citric > lactic > malic > tartaric > HCl. The order for growth-inhibiting properties was the same, except that citric and lactic acids changed places in the series. Levine and Fellers *(13)* noted that acetic acid was much more effective than either hydrochloric or lactic acid against *Saccharomyces cerevisiae* or *Salmonella aertrycke*. Wyeth *(26)* reported that on a pH basis the order of effectiveness against coliform bacteria was acetic > lactic > hydrochloric. Shillinglaw and Levine *(22)* found that in a concentration of 0.02N the order of effectiveness of the edible acids as germicides against *Escherichia coli* at 30° C. was tartaric > lactic > acetic > citric.

The influence of salt. The influence of salt on the viability and thermal resistance of microorganisms has commanded the interest of various investigators who have reported both increased lethality and a definite protective effect. Viljoen *(24)* found that concentrations of sodium chloride up to 3.5 percent in pea liquor gave a protective effect against heat to certain microorganisms including thermophiles. The greatest protection was observed at 1.0-2.5% while 4.0% either gave no protective action or else had a killing effect. Anderson *et al. (11)* showed that the addition of increasing amounts of salt to tomato juice resulted in a progressive increasing of the hydrogen ion concentration and a concomitant more rapid destruction rate for *B. thermoacidurans*.

The influence of sugars. Erickson and Fabian *(8)* demonstrated clearly that thermophiles are much less resistant to the effects of sugars than other microorganisms such as *Streptococcus lactis* and *Streptococcus liquefaciens*. They found, for three cultures of thermophiles, a preservative action in the presence of 17.5% fructose, 27.5% dextrose and 40 to 45% sucrose. Anderson *et al. (1)* found that increasing concentrations of sucrose and dextrose in tomato juice enhanced the thermal resistance of *B. thermoacidurans*. Robertson *(19)* observed an increasing protective action for several microorganisms, including *Streptococcus thermophilus*, when heated in increasing concentrations of sugars. Several workers including Baumgartner and Wallace *(3)* and Fay *(9)* found that the thermal resistance of some microorganisms when heated in hypertonic solutions of sucrose, glucose, lactose and other sugars is increased.

PROCEDURE

The cultures[b] of thermophilic bacteria used in this investigation were: Obligate thermophile that sour No. 26, 1373 and 1503 isolated from corn, hominy and peas respectively, and facultative thermophiles No. 1401 and 1518 isolated from pumpkin and corn respectively. Culture No. 1373 became contaminated during the experiment and was omitted from the later experiments.

The food ingredients whose effect on the viability of the above thermophilic bacteria was studied and their sources are as follows: acids — acetic — Glacial, C.P. grade, citric — Baker's analyzed, C.P. grade, lactic — Edible lactic acid, 50 percent; salt — U.S. sodium chloride, Merck; sugars — dextrose, Bacto dextrose, anhydrous, sucrose, C.P., fructose (levulose) C.P.

Growth curves were run on cultures 26 1401, 1503 and 1518 in order to determine their rate of growth and death in dextrose tryptone broth and the approximate time intervals at which platings were to be done throughout the experiment.

Methods. Fifty ml. portions of sterile dextrose tryptone broth were preheated to 55° C. to reduce optimal lag phase from the very start. Each was inoculated with 1 ml. of a suspension of each organism, washed from a 48 hour dextrose tryptone agar slope and incubated in a water bath maintained at 55 ± 1° C. Plate counts were made at 2 hour intervals up to 20 hours, and at 6 hour intervals up to 48 hours and also at 72 and 96 hours.

A sterility test was run on each batch of acid as follows: A drop of the acid was added to a test tube containing 10 ml. of dextrose tryptone broth. The tube was then incubated at 55° ± 1° C. for 48 hours. In no instance, however, did thermophiles appear in any of the acids used.

Using aseptic precautions throughout all manipulations, sterile dextrose tryptone broth was adjusted with the aid of a Beckman pH meter, to the required pH values. Fifteen ml. portions were then dispensed into sterile test tubes.

Sodium chloride, in concentrations of 0, 0.5, 5, 1.5, 2.5, 5.0, 10, 15, 20 and 25% by volume, was added to dextrose tryptone broth and the mixture heated in the steamer to dissolve all the ingredients. The broth was then dispensed in 15 ml. quantities into test tubes and the tubes autoclaved at 121° C. (250° F.) for 15 minutes.

In the case of dextrose, amounts equivalent to 5, 10, 20, 30, 40, and 50% by volume were added to tryptone broth and the procedure repeated exactly as in the case of the salt solutions. For fructose and sucrose 100% weight by volume solutions were made up and autoclaved at 10 pounds pressure for 10 minutes. Enough of these stock solutions were then aseptically pipetted into sterile flasks to make up 100 ml. of each of the concentrations required. From these flasks 15 ml. portions were then aseptically pipetted into sterile test tubes.

All cultures were grown on dextrose tryptone agar slants for 48 hours. This allowed the use of cultures of a standard age of 48 hours throughout the experiment.

All the tubes of broth for the acid, salt or sugar test were preheated at 55° C. for 2 hours. The 48-hour cells were washed off the dextrose tryptone agar slopes using 10 ml. of sterile distilled water. Into each of the tubes, 1 ml. of the well-shaken cell suspension was inoculated, kept in a water bath maintained at 55 ± 1° C. during this process, and tubes then incubated at 55 ± 1° C.) Plating on dextrose tryptone agar by the pour plate method was done from each tube after 0, 6, 12, 24, 48, and 96 hours of incubation and plates incubated at 55 ± 1° C. for 36 hours. All inoculations of tubes and platings from tubes were done in duplicate.

RESULTS AND DISCUSSION

Figure 1 shows the normal growth curves in dextrose tryptone broth for cultures 26, 1401, 1503, and 1518 after increasing periods of incubation. Figure 2 shows the logarithmic growth curves of 5 thermophiles when grown at 55° C. in dextrose tryptone broth adjusted to pH 5.5 with acetic, citric, and lactic acids respectively. Figure 3 shows the logarithmic growth curves of 5 thermophiles, viz., No.'s 26, 1373, 1401, 1503, and 1518, when grown at 55° C. in dextrose tryptone broth to which various amounts of sodium chloride had been added. Figure 4 shows the logarithmic growth curves of 4 thermophiles, viz., No.'s 26, 1401, 1503, and 1518, when grown at 55° C. in tryptone broth to which different percentages of dextrose, fructose and sucrose respectively had been added.

From Figure 1 it is seen that in all cases maximum growth was evidenced after 8 to 14 hours of incubation. This was followed by a very rapid decrease in viable numbers which, in several cases, were much lower after 96 hours of incubation than the initial inoculum.

Gaughran *(7)*, Insecticki and Schizeve *(11)*, and Hanson *(10)* all noted that the growth of thermophilic bacteria begins almost immediately on incubation in the culture medium, the initial lag phase being either absent or too short to be detectable. Both growth and death rates were found to be much more rapid than among the mesophiles, i.e. the generation time is much shorter and the logarithmic growth phase during which

[b] Obtained from the National Canners Association.

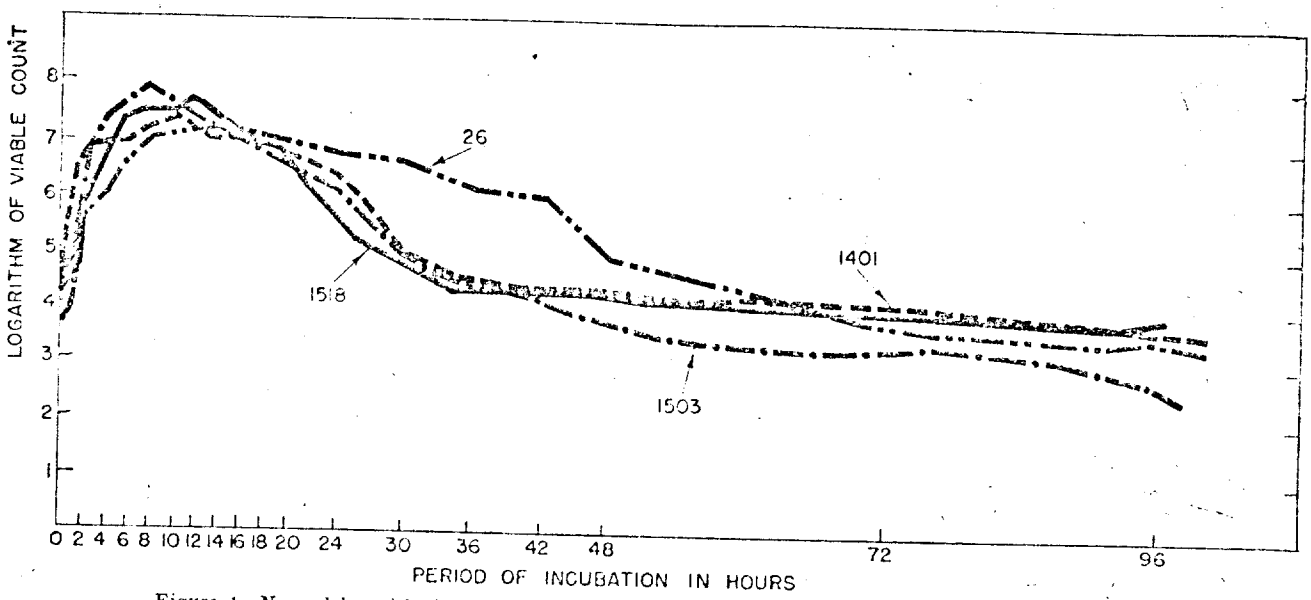

Figure 1. Normal logarithmic growth curves of test organisms in dextrose tryptose broth at 55° C.

the rate of multiplication remains constant was not evident in population curves. Imsenecki and Solnzeva *(11)* also noted that proteolysis, denitrification and hydrolysis of starch by thermophilic bacteria proceed at a rate seven to fourteen times that of cultures of mesophilic bacteria. Such rapid multiplication and death rates of cells have been attributed to: (a) The extremely high biochemical activity of thermophilic bacteria as a result of their rapid growth; (b) The inability of thermophilic bacteria to sporulate readily due to the very low oxygen tension in the liquid medium at such an elevated temperature *(7)*; (c) The rapid formation and accumulation of acids through the decomposition of carbon compounds in the medium if such are not neutralized, since the fermentation capacity of thermophiles has been calculated to be perhaps thirty times as great as that of *Streptococcus lactis* at 20° C.; (d) Cytolysis induced by autolysis with the resultant accumulation of toxic products in the medium. Evidence of autolysis has been obtained by way of the detection of an accumulation of enzymes in media inoculated with thermophiles *(11)*.

The effect of increasing concentrations of acids, salts, and sugars was determined in terms of percent increase or decrease of cell numbers relative to the initial inoculum. Results are recorded in Figures 2, 3, and 4. From Figure 2 it may be noted that at pH 5.5 the influence of the acids on growth follow the order citric > acetic > lactic.

In results not shown here when the acidity was increased by citric acid beyond pH 6.0 down to pH 5.5 there was decreased growth. Beyond pH 5.5, however, an increase in acidity resulted in no significant decrease in the number of viable cells. For acetic and lactic acids, multiplication occurred at pH 5.5 and germicidal action started at pH 5.0. A decrease in pH below pH 5.0 afforded no significant decrease in viable cells.

Several investigators have attributed the effect of added acids in suppressing the growth of bacteria to the change in the hydrogen ion concentration. However, Erickson and Fabian *(8)*, Anderson *et al.* *(1)*, Nunheimer and Fabian *(15)*, and Levine and Fellers *(13)*, have pointed out that the toxicity of certain organic acids is due in part to the undissociated molecule and the anion, in addition to the effect of the hydrogen ion.

Cohen and Clark *(6)* suggested that the action of acetic acid may be due to the free acetate radical exerting a synergistic effect upon the disinfecting power of the hydrogen ion.

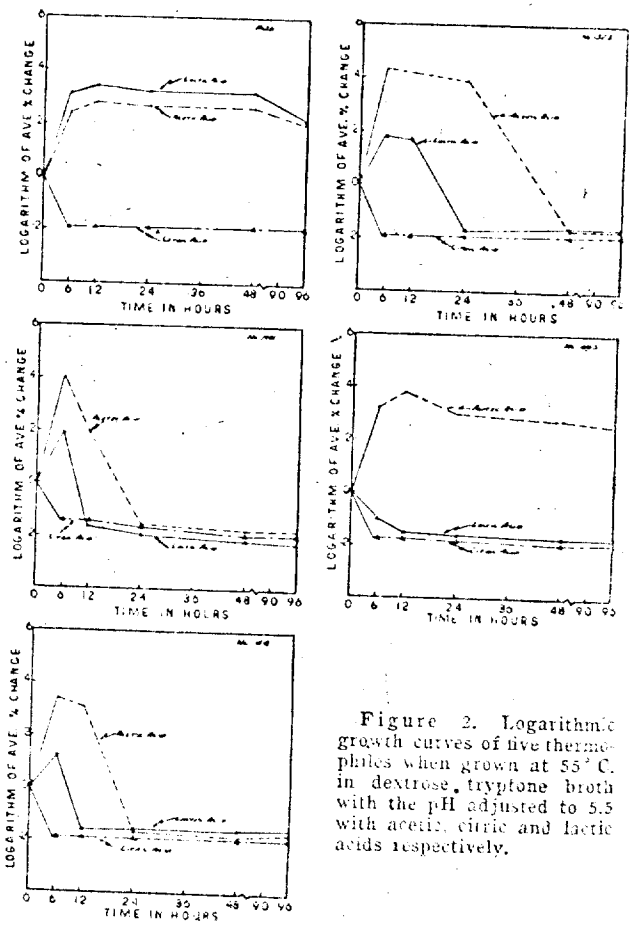

Figure 2. Logarithmic growth curves of five thermophiles when grown at 55° C. in dextrose, tryptone broth with the pH adjusted to 5.5 with acetic, citric and lactic acids respectively.

Reid *(18)* believes that the anion and undissociated molecule may act independently in exerting their disinfectant action, or as positive catalysers increasing the bactericidal power of the hydrogen ions.

The influence of acids on the surface tension of the medium and the size of the molecule have been mentioned by Bach *(2)* as possible reasons for observed germicidal action. He also stated that the hydrogen ions control the antiseptic effect but the undissociated part of lactic acid is the germicidal factor when the pH value is important.

Traube's rule *(14)* states that in an homologous series of fatty acids the lowering of surface tension is parallel to the increase in molecular weight of the fatty acid. This may explain why citric acid is more germicidal than either acetic or lactic since of the three acids citric has the largest molecular weight. The dissociation of the acids may also account for the order of germicidal action observed.

If dissociation was the prime factor in the germicidal action then the order should be citric > lactic > acetic. This again may explain why citric acid was more toxic than either acetic or lactic acid. Since acetic acid was found to be more germicidal than lactic acid the undissociated molecule may be toxic as suggested previously.

From Figure 3 it may be seen that up to 1.5%, sodium chloride was non-germicidal except in the case of organism No. 1503. For No. 1503 germicidal action took place at 1.5% but not at 0.5%. Concentrations of 2.5% or greater proved germicidal in all cases. With culture No. 26 total destruction took place at 5.0% and greater.

The reason for the action of salt in inhibiting the growth of bacteria or destroying the cells is not readily apparent. Anderson *et al. (1)* observed that there is obviously no toxic entity in salt. Moreover, as prementioned, certain investigators including Viljoen *(21)* have attributed a protective effect from heat on some microorganisms by certain concentrations of salt. Anderson and his colleagues think that, inasmuch as the addition of increasing amounts of salt to media is known to result in increased hydrogen ion concentrations, the lethal effect of salt may be due at least in part to the lowered pH of the substrate.

The effect of salt on bacteria is thought by many investigators to be mainly and simply plasmolytic. Plasmolysis, however, is believed to last for only a few hours and not all bacterial cells are affected.

Rodwell and Ebertz *(20)* suggested that at least five factors are involved in the detrimental action of salt on bacteria, namely: (a) The direct effect of the chloride ion; (b) Dehydration; (c) The removal of oxygen; (d) Sensitization against carbon dioxide; (e) Interference with the rapid action of proteolytic enzymes.

In addition to the above postulates it may be that, due to the dissociation of sodium chloride in solution, the sodium and chloride ions, though necessary for the growth of some organisms, and accelerators in small amounts, become toxic in large quantities, i.e., as the concentration of salt is increased. This is an established fact in bacterial physiology. The law of diminishing returns seems evident as the concentration of salt gets

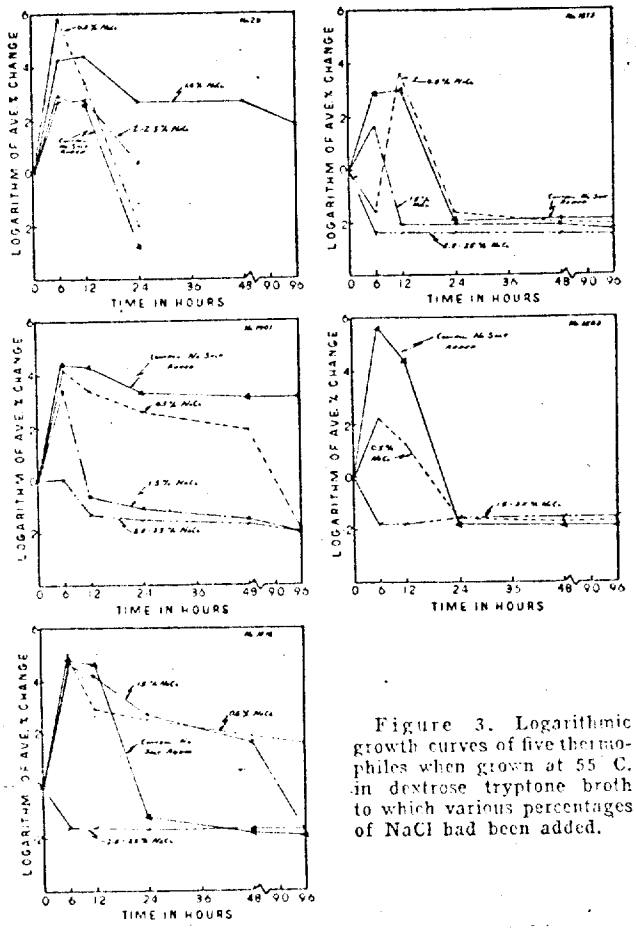

Figure 3. Logarithmic growth curves of five thermophiles when grown at 55° C. in dextrose tryptone broth to which various percentages of NaCl had been added.

beyond 2.5% since even a concentration of 2.5% gave no significant increase in germicidal action.

From the average percentages of increase or decrease over the initial inocula in the presence of varying concentrations of dextrose, fructose and sucrose shown in Figure 4, it may be seen that 5.0% dextrose did not suppress growth over the first 6-12 hours of incubation in the case of cultures 26, 1373, and 1518. A decrease of 66% occurred after 12 hours in culture 1503. For culture 1401 a decrease of 72% took place after 6 hours. A 10% solution of dextrose showed germicidal action for all organisms after 6 hours.

During incubation periods of 6-12 hours multiplication took place in the presence of 10.0% fructose. Concentrations of 20% or greater proved germicidal in all cases.

Except for organism 1401 sucrose in equivalent concentrations gave results in the same trend as fructose. Sucrose, however, was less destructive beyond 20.0%. The reverse of this holds true for organism 1401.

For all three sugars, increasing the concentration beyond that needed for germicidal action showed no increased lethality. The effectiveness of the sugars in reducing the bacterial count may therefore be written as dextrose > fructose > sucrose.

The reasons for the germicidal action of sugars against microorganisms are not well established. Concentrations of 10-50% of sucrose and dextrose are known to cause no appreciable change in the pH of cul-

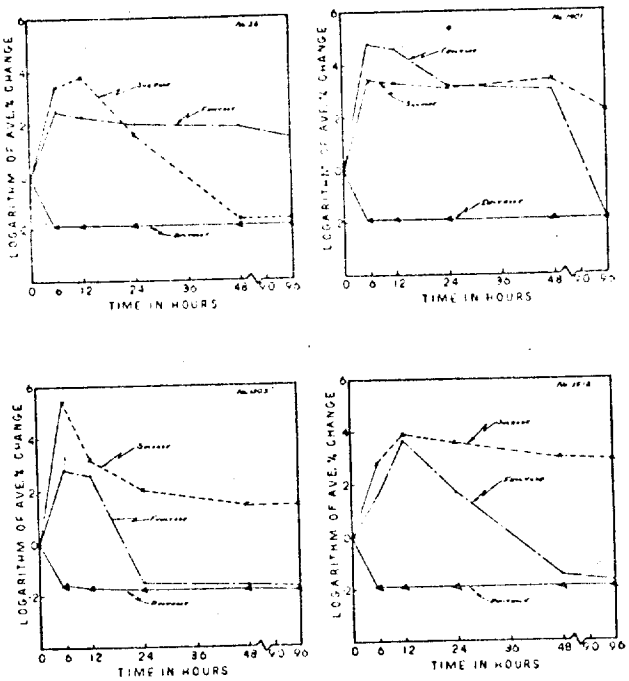

Figure 4. Logarithmic growth curves of five thermophiles when grown at 55° C. in tryptone broth to which different percentages of dextrose, fructose and sucrose, respectively, had been added.

ture media, hence no significant portion of the germicidal action of the sugars can be attributed to pH changes.

Fay *(9)* thinks that in both the protective and germicidal actions the influence of sugars on osmotic pressure determines their effectiveness. In his opinion the permeability of the individual cell apparently plays an important part in regulating the rate of death in water and in sugar suspensions. He hypothesi-es that resistance to the effects of sugars is limited to those cells which are highly sensitive to water at slightly increased temperatures.

Plasmolysis of the microbial cell may explain why dextrose exerted a germicidal effect at a lower concentration than either fructose or sucrose and why fructose exerted a greater germicidal action at the same concentration as sucrose. Osmotic pressure and hence plasmolysis depends on the number of particles in solution. Dextrose and fructose each with a molecular weight of 180 would, therefore, contain more molecules per unit of weight than would sucrose. The activity should, therefore, tend to decrease as the molecular weight increases. On a chemical basis, dextrose, an aldehyde sugar, should be less reactive than fructose, a keto sugar, as evidenced by the fact that many chemical tests can be carried out on the latter in the cold while the former must be heated. This order of activity apparently does not hold biologically in these experiments since dextrose was found to be more germicidal than fructose. The reason for this is not apparent. Furthermore, the results obtained here are contrary to those obtained by Erickson and Fabian *(10)* who found that the order of effectiveness was fructose > dextrose > sucrose > lactose.

SUMMARY AND CONCLUSIONS

The growth of thermophilic bacteria was found to be very rapid reaching a maximum within 8-14 hours, followed by a rapid decline in cell numbers. The lag phase of the growth cycle is not very pronounced and after 96 hours there were fewer viable cells present than in the initial inocula.

The order of germicidal action of the acids studied is citric > acetic > lactic. An increase in acidity beyond pH 5.0 gave no increased germicidal action.

Concentrations of sodium chloride up to 1.5% were not germicidal except for organism No. 1401 which was affected at this concentration. Concentrations of 2.5% and greater were germicidal in all cases. An increase in salt concentration beyond 2.5% gave no added germicidal effect.

The order of effectiveness of the sugars as germicides was found to be dextrose > fructose > sucrose. In all cases concentrations beyond 20.0% gave no significant increase in germicidal action.

From the growth curves it may be concluded that the deleterious action of various food ingredients on thermophilic bacteria occurs during the first 6 to 12 hours of incubation. This interval corresponds roughly to the phase of physiological growth and the early logarithmic phase of the bacterial growth curve.

LITERATURE CITED

1. Anderson, A. A., *et al.* Effects of acids, salt, sugar and other ingredients on the thermal resistance of *Bacillus Thermoacidurans*. *Food Research*, 14, 499-509 (1950).
2. Bach, D. Antiseptics. *Bull. Sci. Pharm.*, 39, 499-509 (1932).
3. Baumgartner, J. G., Wallace, M. D. The destruction of microorganisms in the presence of sugars. *J. Soc. Chem. Ind.*, 53, 2941-2971 (1934).
4. Bergey, D. H. Thermophilic bacteria. *J. Bact.*, 4, 301-306 (1919).
5. Cameron, E. J., and Esty, J. R. The examination of spoiled canned foods. Classification of flat-sour spoilage organisms from non-acid foods. *J. Inf. Dis.*, 39, 89-106 (1926).
6. Cohen, B., and Clark, W. M. The growth of certain bacteria in media of different hydrogen ion concentration. *J. Bact.*, 4, 409-424 (1919).
7. Cratchman, Eugene R. L. The Thermophilic Microorganisms. *Bact. Reviews*, 11, 189-225 (1947).
8. Erickson, F. J., and Fabian, F. W. Preserving and germicidal action of various sugars and organic acids on yeasts and bacteria. *Food Research*, 7 (1), 168-79 (1942).
9. Fay, A. C. The effect of hypertonic sugar solutions on the thermal resistance of bacteria. *J. Agri. Res.*, 48 (5), 453-473.
10. Hansen, A. The growth of thermophilic bacteria. *Arch. Microbiol.*, 23 (1933).
11. Imshenecki, A., and Sinezeva, L. Growth of aerobic thermophilic bacteria. *J. Bact.*, 49, 539-546 (1945).
12. Jones, A. H., and Fabian, F. W. The viability of microorganisms isolated from fruits and vegetables when frozen in different menstrua. *Tech. Bull.* 229, Mich. Agr. Exp. Station (May, 1952).
13. Levine, A. S., Fellers, C. R. Action of acetic acid on food spoilage microorganisms. *J. Bact.*, 38, 499-513 (1939).
14. Maclean, C. *Cell of Science*. 1950, D. C. Heath and Company, New York, N. Y.
15. Nesbitt, T. D., and Fabian, F. W. Influence of organic acids, sugars and sodium chloride upon strains of food poisoning staphylococci. *Am. J. Pub. Health*, 30, 1040-1049, 1940.

16. PEDERSON, C. S., AND BECKER, M. E. Flat sour spoilage of tomato juice. N. Y. State Agr. Sta. Tech. *Bull.*, 287, 1-22 (1949).

17. PRICKETT, P. S. Thermophilic and thermoduric microorganisms with special reference to species isolated from milk. Description of spore forming types. N. Y. Agr. Exp. Sta. *Tech. Bull.*, 147 (1928).

18. REID, JAMES A. The disinfectant action of certain organic acids. *Am. J. Hyg.*, 16, 549-556 (1932).

19. ROBERTSON, A. H. Thermophilic and thermoduric microorganisms with special reference to species isolated from milk. Vermont Agr. Exp. Sta. *Bull.*, 274, 27 (1927).

20. ROCKWELL, GEORGE E., AND EBLERTZ, EDWIN G. *J. Infections Diseases*, 35, 573-575 (1922).

21. SCHMIDT, CLARENCE F. Spore formation by thermophilic flat-sour organisms. The effect of nutrient concentration and the presence of salts. *J. Bact.*, 60 (2), 205 (1950).

22. SHILLINGLAW, C. A., AND LEVINE, MAX. The effect of acids and sugars on the viability of Escherichia coli, and Eberthella typhosa. *Food Research*, 8 (6), 464-476 (1943).

23. TANNER, F. W., AND WALLACE, G. I. Relation of temperature to the growth of thermophilic bacteria. *J. Bact.*, 10, 421-437 (1925).

24. VILJOEN, J. A. 1—Heat resistance studies. 2—The protective action of sodium chloride on bacterial spores in pea liquor. *J. Infectious Diseases*, 39, 286-290 (1926).

25. WALLACE, G. (Thesis, University of Illinois, 1924).

26. WYETH, F. J. S. The effect of acids on the growth of *Bacillus coli*. *Biochem. J.*, 12, 382-401 (1918).

Alterations and Reactions Induced in Normal Tissues by Lytic Substances (Acetic Acid, Nitric Acid)
1. Effects on Vascular-Neural Bundles

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Introduction

Of special interest to surgeons and oncologists is the problem of dissemination of tumor cells during a surgical operation, the breaking in of these cells into the blood and lymphatic stream, and their dispersion in the operating area itself.

Numerous studies indicate the presence of tumor cells in the blood of patients undergoing surgical operation for cancer. For example, J. F. Potter et al. (1960) have found that such cells are present in 29% of the cases, while J. C. Pruitt (1962) found them present in 30% of the cases. The percentage of tumor cells increases when the blood of veins draining the tumor is examined, and may reach a value as high as 43%, according to J. F. Potter, especially when blood sampling is performed during the course of the operation (M. Dargent et al. 1963, A. Prudente 1963). It should be noted that whereas the presence of tumor cells in the blood does not seem to be of great importance in regard to prognostic effects, according to H. C. Engell (1955), M. Dargent et al. (1963) and V. Pasqualucci et al. (1964), it would appear to be highly important in regard to eventual metastases, according to D. R. Fisher and R. B. Tunbull (1955), G. E. Moore (1960) and A. Prudente (1963).

As far back as 1867, C. H. Moore has called attention to the propagation of the tumor on the operation wound; later, Ryall (1907), W. H. Cole et al. (1954), E. A. McGrew et al. (1954), R. R. Smith et al. (1958),

A. Peracchia and E. Fasani (1960) and others have also shown the damages caused by contamination and have suggested measures for avoiding such contamination. Tests carried out by R. R. Smith et al. in 1953, and then repeated 8 years later, have made it possible to note that washing water in the operating area contains tumor cells in 25.9% of the cases. The drainage of wounds has also shown that tumor cells are present at long as 72 hours after the surgical operation. By adding up the positive cases observed after washing and drainage, the percentage of tumor cells was found to increase later on by one-third (R. R. Smith, 1963).

The histological type also is of slight importance in the way relapses in situ are determined. According to R. R. Smith, in fact, relapses of mucous cancer exceed 40% in short time and after 5 years even amount to 53%.

Various technical measures have been suggested for neutralizing the dissemination of neoplastic cells during surgical operations, which may later lead to relapses in situ or to metastases; such measures include washing the operating area after surgery. The products used so far for this purpose (saline solutions, alcohol, ether, formaldehyde, etc.) have not yielded, however, the results hoped for.

The idea has also occurred of using, during the operation itself, some strong acids in appropriate dilution by local topical application, in an attempt to destroy tumor cells without inflicting secessive damage to healthy tissues.

Prior to testing such caustic (corrosive) products on neoplastic tissues, we wanted nevertheless to check the way in which various acid solutions would act on normal tissues, and particularly on vascular-neural bundles.

General Considerations

The clinical use of chemical substances for washing the operating area is probably as old as surgery itself, and many surgeons used to wash wounds systematically with various solutions. The products most frequently used are saline solutions, alcohol, ether, a 0.5% formaldehyde solution, a formaldehyde-picric acid mixture (R. R. Smith, 1963), antimitotic compounds (W. H. Cole et al., 1954), nitrogen mustard (A Prudente, 1963), and sodium hypochlorite (F. A Mukhtar et al., 1963); nevertheless, the results

obtained are quite contradictory. It was noted by R. R. Smith et al. that, in those cases where the search for tumor cells in the washing water used in the operating area gave negative results, the application of local chemotherapy led to a definite decrease of relapses. On the contrary, in those cases when neoplastic cells were found in the washing water a high percentage of relapses was noted, even after the topical use of chemotherapy in the operating area.

Recently, research on substances capable of destroying tumor cells has increased again: thus, J. C. Maruzzella and B. I. Brenner (1963) have tested 50 mineral oils; F. A. Mukhtar et al. (1963) have tested in turn 60 products, including 9 dyes, 8 oxidizing agents, 6 pyridine derivatives, 3 triazine compounds, 8 alkylating agents, 7 carbamate chelating agents and 11 various products (hydrazine, porphyrin, etc.). After selecting a few substances which had yielded encouraging results in vitro, the above authors have experimented with the local application of nitrogen mustard and sodium hypochlorite; however, a larger number of relapses was noted after the use of such products. Better results have fortunately been reported by W. H. Cole et al. (1954), who irrigated the operating area with antimitotic compounds.

Personal Experimentation

a) Formulation of the problem.

The aim of our studies was to test some chemical products which are easily available commercially, and which would be able to destroy any eventual fragments of tumor tissue that remain and can be detected in the operating area during surgery.

For experimental purposes, two strong acids were considered: glacial acetic acid and nitric acid in various concentrations; the effect of these acids on protein substances is well known. In order to determine which concentration is the most suitable for causing a superficial coagulation of protein substances without damaging the underlying tissues, it was believed that the solutions of the acids examined shoud be tested for their effect on very delicate tissues. Therefore, these solutions were applied by brushing on vascular-neural bundles located at the roots of front and rear limbs of medium-size rabbits, in such a way that any injurious effects that might be noted could be clearly visible and reported.

b. Materials and methods

In all our studies, 20 rabbits of medium weight were used, subdivided into 5 groups, along with a control series of 3 rabbits.

Glacial acetic acid was tested as a N/10 (0.1N) solution, a normal solution (N), and as a 25% and 50% solution. Nitric acid was tested as a normal (N) solution, and as a N/10 (0.1N) solution.

In each animal, the solution studied was applied by brushing all 4 vascular-neural bundles and neighboring tissues, after they had been exposed under local anethesia. The skin wounds were then accurately sutured, naturally with all operations done under sterile conditions, and no other treatment was used. The rabbits were put back into their cages and kept on a normal diet together with untreated controls. Four rabbits in each group were sacrificed 30-35 days after the operation.

c. Macro and microscopic observations

During the post-operational observation period, no behavioral disorder was noted in the rabbits; mobility, rest, appetite and digestion were also normal, and the weight gain followed a normal course. None of the rabbits subjected to the operation died, and all wounds healed normally as usual. After the animals were sacrificed, the entire axillary and iliac regions were removed as a unit, including cutaneous, subcutaneous and muscular tissues and the vascular-neural bundles.

In the areas which had been in contact with the acid solution studied, adhesion was noted between cutaneous and subcutaneous layers and the muscular-fascial planes; the presence of a whitish, scarcely firm tissue was noted, which was almost gelatinous due to intense edema and much more abundant in rabbits treated with solutions of higher concentration; this alteration extended over the entire area treated with the acid solution. The vascular-neural bundles were easily recognizable even when coated by edema, which was considerable (marked) in those cases where 25% and 50% acetic acid and normal nitric acid solutions were used. The elements adhered to each other at their base, and could be separated; no apparent vascular thromboses were noted. In those cases treated with acids of higher concentration, and especially with nitric acid, one had the impression that the walls were thickened, due to their greater firmness to touch.

Various histological samples were prepared from the tissues collected, and their observation has made it possible to make some interesting observations, which are reported below.

First group: rabbits treated with N/10 acetic acid.

The striated muscular tissue exhibits on its periphery degenerative symptoms of various degree: the myocellular elements in some areas show a tendency to swell and lose their own specific coloring property; in other areas, on the contrary, they exhibit a more advanced picture (pattern), in which the cytoplasm becomes opaque and assumes a granular appearance, and cellular lysis phenomena are also noted, with some extreme patterns of cellular necrosis (Fig. 1.). In the middle of this muscular context (pattern), the vascular-neural bundle is well recognizable. Arterial vessels show a moderate thickening of the adventitia (Fig. 2.); the veins present a light state of subintimal edema (Fig. 3.). The same edematous state with diastases of neural fibers can be detected in the neural trunks (Fig. 4.). The perivascular fatty connective tissue is undamaged.

Second group: rabbits treated with a normal acetic acid solution.

The striated muscular tissue shows slightly more pronounced alterations, as described above, but always limited to peripheral areas. The artery exhibits a moderate thickening of the adventitia. The neural trunks, although appearing undamaged in the central portion, exhibit superficially (on the surface) signs of edema and swelling of neural fibers, which often appear, on transverse sections, as optically void small disks, adjacent in the middle to highly edematous areas (Figs. 5-9.).

Third group: rabbits treated with a 25% acetic acid solution.

Fragments consist primarily of fibrous connective tissue. The myocellular bundles exhibit more pronounced regressive phenomena, which reach the level of cellular lysis and substitution by fibrocytic elements. The arterial and venous vessels appear thickened, especially in the intimal and subintimal zone. However, the component showing the most marked regressive phenomena is the neural (nerve) component, in which the bundles are separated due to edema. Aa a result, single fibrils are swollen, with a balloon-like appearance on transverse section, and of variable degree

which can extend up to lysis. These degenerative phenomena do not encompass the whole nerve, but are limited to groups of fibers located at the periphery (Figs. 10-14).

Fourth group: rabbits treated with a 50% acetic acid solution.

Alterations here are even more pronounced. Microcellular elements undergo extensive regressive phenomena which, passing through phases of cytoplasmic opacity and paste formation, almost reach the stage of complete cellular lysis (dissociation). There is an increase in fibrous stroma which takes the place of muscular elements, thus giving rise to a tight grid in the middle of which residual and still preserved small myocellular bundles remain enclosed. The vessels appear with thickened walls due to a fibrosis and thickening of the adventitial and periadvential zone. In these preparations, elements located for from contact with the acid solution tested also remain normal or almost normal (Figs. 15-18).

Fifth group: rabbits treated with a N/10 nitric acid solution.

Myocellular bundles exhibit regressive phenomena consisting in a swelling, opaqueness and homogenization of single fibrous cells. The vessels sometimes appear thickened, but more often exhibit small intramural, necrobiotic foci. Moderate phenomena of periadventitial fibrosis are noted. Small neural bundles also exhibit regressive phenomena, which mainly caused by the edematous condition and dissociation of fibers (Figs. 19-23).

Sixth group: rabbits treated with a normal nitric acid solution.

All the various tissue elements are affected in a degenerative sense. The neural trunks appear with fibers greatly dissociated and swollen fibers, due to edema, and sometimes with diffuse, neurolytic phenomena. In transverse sections, single fibrils are reduced to small roundish and optically void rings. Vessels exhibit swollen, homogeneous and thickened walls with fibrosis of the adventitial layer. The striated muscular component also exhibits partial regressive phenomena up to the stage of cellular lysis. The destroyed muscular tissue is replaced by fatty (adipose) tissue or, in smaller amounts, by newly formed fibrous connective tissue (Figs. 24-29).

Summary Results

During the 30-35 days of observation, all test animals behaved in the same ways as the animals of the control group: their movements, appearance, rest, digestion and weight gain were normal. No rabbits died prior to being sacrificed.

Macroscopically, the tissues treated exhibited a certain degree of mutual adhesion and or more or less greater amount of newly formed fibrous tissue, according to the concentration of the acid solution used.

Histologically, the differences were quite noticeable.

a) Acetic acid. The tissues which are most sensitive to less concentrated solutions, such as the N/10 solution, are muscular tissues: the myocellular elements are swollen, lose their staining properties or exhibit a granular appearance up to the stage of cellular lysis. At a higher concentration, such as the normal solution, even nerve fibers in the peripheral tract exhibit phenomena of edema and swelling. With more concentrated solutions, major injuries are noted, with cellular lysis of both muscular fibers and nerve fibers. On the other hand, both venous and arterial vessels show slight signs of reaction, with a thickening of the adventitia and periadventitial tissues.

b) Nitric acid. Injuries definitely more severe were noted in the two groups of rabbits treated with N/10 and normal acid solutions. Degenerative alterations and even necroses of the muscle cells were observed; edema fiber diastasis and necroses of nerve fibers were noted. In regard to arteries and veins, a thickening of the intimal and subintimal zone was observed, and occasionally small areas of necrobiosis.

Conclusions

Nitric acid solutions, even those greatly diluted, inflict damage not only to muscular fibers, but also to nerve fibers, whereas normal and N/10 acetic acid solutions spare (do not injure) vessels and nerves, causing irreversible alterations only in muscular elements. This observation allows us to assume that any tumor cells eventually present and disseminated in the operation area during surgery, being very vulnerable during accelerated mitosis, could be easily destroyed if they came into contact with the above acid solutions. Therefore, in view of the good resistance of vascular-neural bundles, it would appear possible to apply a normal or N/10

solution of acetic acid by brushing the operation area, after removal of the tumor. For example, following a radical mastectomy, the application by brushing of such solutions could possibly destroy any eventual tumor tissues remaining in the operation area, which often are the cause of relapses.

Subsequent clinical experience will show whether such experimental assumptions will prove to be useful in any way.

Microphotographs of histological preparations based on findings obtained from rabbits treated with N/10 acetic acid. First Group.

Fig. 1. Fragment of striated mussels: swelling and granular degeneration of fibers. Some phenomena of chopping (breaking into small pieces) of the fibers proper.

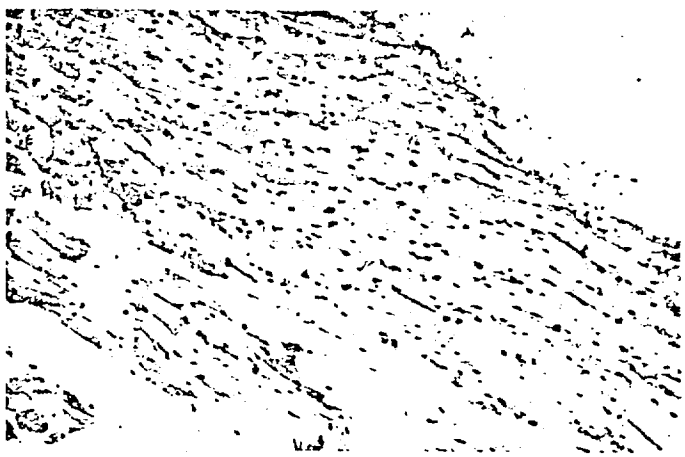
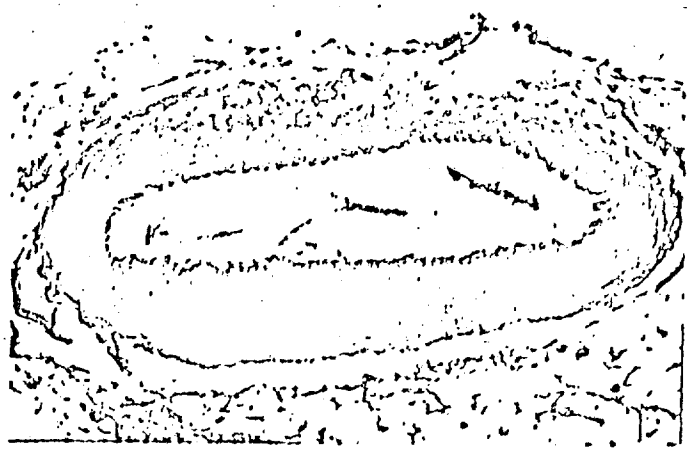


Fig. 2. Artery: fibroses of the adventitia.



First group: rabbits treated with N/10 acetic acid solution.

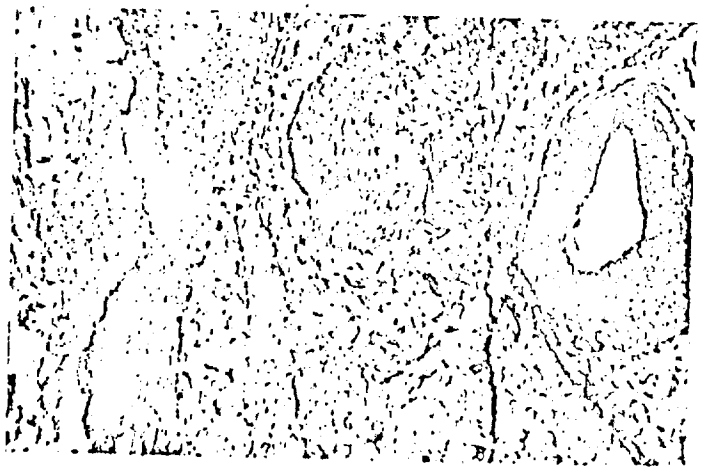
Fig. 3. Peripheral vein: some phenomena of intimal swelling with protusion in the lumen.

Fig. 4. Peripheral neural ramification: fascicular (bundle) edema.

Second group: rabbits treated with a normal acetic acid solution.

Fig. 5. Fragments of muscular fibers: interstitial dissociation due to edema and turbid (cloudy) degeneration of fibers, primarily of peripheral fibers.

Fig. 6. Vascular formations in cellular-adipose tissue. Adventitial fibroses affecting arteries and veins.



Second group: rabbits treated with a normal acetic acid solution.

Fig. 7. Details of preceding figure (Fig. 6): adventitial, periarterial fibroses.

Fig. 8. Peripheral nervous (neural) ramification: edema and swelling of fibers located at the periphery of the nerve bundle, observed in a longitudinal section.

Second group: rabbits treated with a normal acetic acid solution.

Fig. 9. The same area (as in Fig. 8), observed in a transverse section of the nerve trunk.

Third group: rabbits treated with a 25% acetic acid solution.

Fig. 10. Fragments of muscular fibers: granular turbid (cloudy) degeneration and fibrous substitution.

Third group: rabbits treated with a 25% acetic acid solution.

Fig. 11. Nerve ramifications: marked dissociative edema.

Fig. 12. Details of preceding figure: balloon-shaped degeneration of nerve fibers and lysis.

Third group: rabbits treated with a 25% acetic acid solution.

Fig. 13. Peripheral artery: proliferative endo- and periarteritis in fibrous evolution, with noticeable reduction of the vascular lumen.

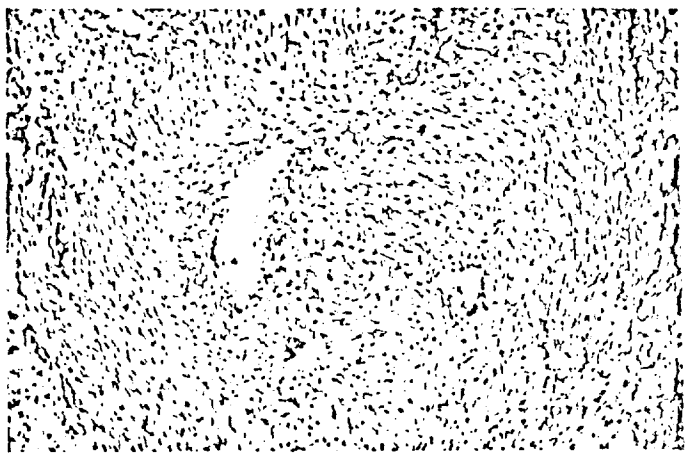


Fig. 14. Peripheral vein: fibrous periphlebitits.

Fourth group: rabbits treated with a 50% acetic acid solution.

Fig. 15. Fragments of muscular fibers: severe signs of muscular degeneration with marked fibrous substitution.

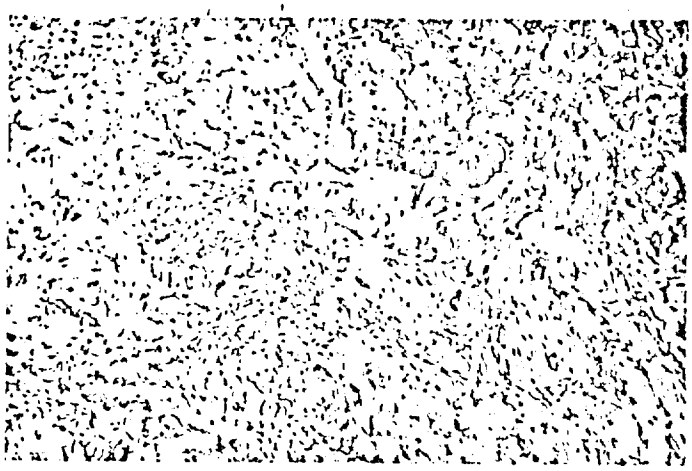


Fig. 16. Peripheral artery: noticeable adventitial and periadventitial fibrosis.

Fourth group: rabbits treated with a 50% acetic acid solution.

Fig. 17. Nerve ramifications: perineural edema and fascicular fibroses. Note the interstitial sclerosis which surrounds the cellular-adipose tissue

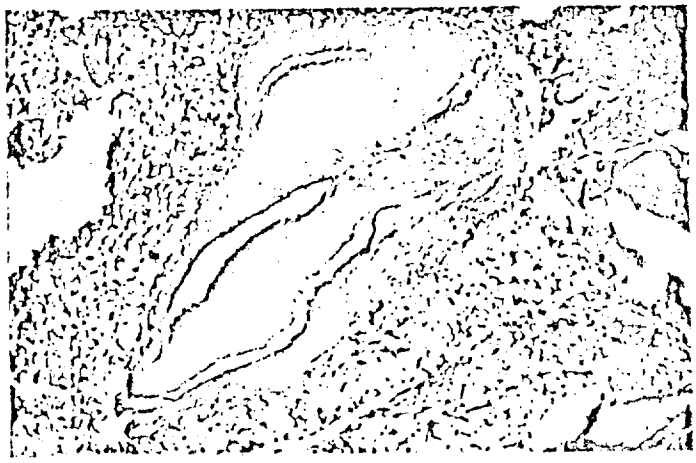


Fig. 18. Details of the preceeding figure, showing the perineural edema and the fascicular fibrosis of the peripheral nerve.

Fifth group: rabbits treated with an N/10 nitric acid solution.

Fig. 19. Peripheral artery: thickening of the middle portion and adventitial fibroses.

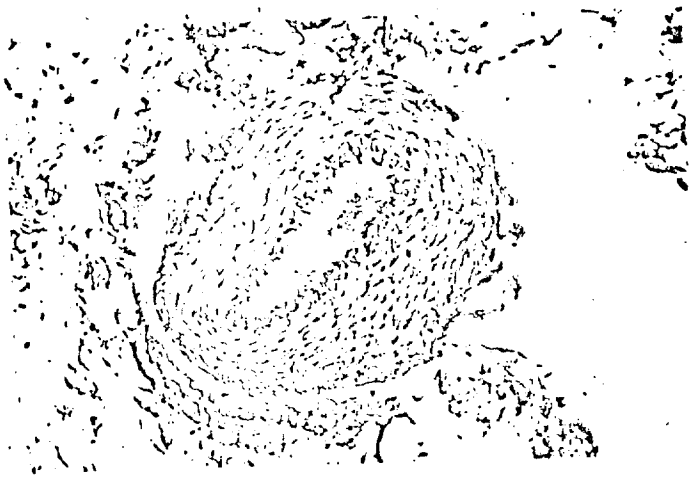


Fig. 20. Artery in longitudinal section at a subdivision point: fibroses of the middle portion and adventitial fibroses.

Fifth group: rabbits treated with an N/10 nitric acid solution.

Fig. 21. Details of preceding figure.

Fig. 22. Venous Wall: sclerotic periphlebitis.

Fifth group: rabbits treated with an N/10 nitric acid solution.

Fig. 23. Peripheral nerve ramifications: dissociative edema of fibers, primarily of those located at the periphery.

Sixth group: rabbits treated with a normal nitric acid solution.

Fig. 24. Fragments of muscular fibers: Marked (extensive) dissociative edema with turbid (cloudy) degeneration up to the stage of fiber lysis.

Sixth group: rabbits treated with a normal nitric acid solution.

Fig. 25. Peripheral artery: moderate thickening of the medium portion.

Fig. 26. Details of preceding figure, showing partial regressive phenomena of the wall.

Sixth group: rabbits treated with a normal nitric acid solution.

Fig. 27. Another arterial ramification: fibrosis of the medium portion and adventitial fibroses.

Fig. 28. Nerve ramifications: perineural and interfascicular edema, with signs of neurolysis.

Sixth group: rabbits treated with a normal nitric acid solution.

Fig. 29. Details showing perineural edema and the balloon-shaped degeneration of some fibers located at the periphery of the nerve trunk.

Summary

The author calls attention to the dissemination of tumor cells during surgical operations and advances the hypothesis of a possible destruction of such cells by irrigation of the operational area with appropriate drugs.

The author has applied various solutions of acetic and nitric acid by brushing the vascular-neural bundles of the limbs of 20 rabbits, in order to determine which concentrations are the most suitable for producing irreversible changes in superficial (surface) cells, without damaging the vascular-neural (nerve) elements. Normal and N/10 solutions of acetic acid fulfill this purpose.

These experiments would indicate that certain lytic substances, such as solutions of acetic acid, could possibly be used by means of local topical application on neoplastic tissues which have remained disseminated and evident (detectable) in the operating area, after surgical removal of the original tumor.

Bibliography

COLE W. H., PACKARD D., SOUTHWICK H. W. – Carcinoma of the colon in relation to recurrence. *J.A.M.A.*, 155, 1549, 1954.

DARGENT M., FAUCON M., CONTI C., POMMATAN E., MAYER M. – Problème des cellules cancereuses dans le sang circulant. *Acta Un. Int. Canc.*, 19, 1095, 1963.

ENGELL H. C. – Cancer cells in the circulating blood. A clinical study on the occurrence of cancer cells in the peripheral blood and in venous blood draining the tumor area at operation. *Acta Chir. Scandin.*, Suppl. 201, 1955.

FISHER D. R., TURNBULL R. B. The cytologic demonstration and significance of tumor cells in the mesenteric venous blood in patients with colorectal carcinoma. *Surg. Gyner. Obst.*, 100, 102, 1955.

MARUZZELLA J. C., BRENNER D. I. – Cit. da SMITH R. R. e Coll. 1963.

MC GREW E. A., LAWS J. F., COLE W. H. – Free malignant cells in relation to recurrence of carcinoma of colon. *J.A.M.A.*, 154, 1251, 1954.

MOORE C. H. – Cit. da MUKHTAR F. A. e Coll., 1963.

MOORE G. E. – The significance of cancer cells in the blood. *Surg. Gyn. Obst.*, 110, 360, 1960.

MUKHTAR F. A., HOFFMEISTER F. S., DI PAOLO J., BROSS I., SHEDDEP W. H., MOORE G. E. – Studies on topical chemiotherapy of tumor contaminated surgical wound. *Am. J. Surg.*, 106, 768, 1963.

Bibliography (con't)

PERACCHIA A., FESANI F. – Chemioterapia del carcinoma della mammella con trietilentiofosforamide. *Tumori*, 46, 205, 1960.

PASQUALUCCI V., DESANCTIS C., TRISTAINO B. – Significato e limiti della ricerca di cellule tumorali nel sangue periferico. *Ann. It. Chir.*, 41, 722, 1964.

POTTER J. F., LONGENBAUGH G., CHU E., DILLON J., ROMSDAHL M., MALMGREN A. – The relationship of tumor type and resectability to the incidence of cancer cells in blood. *Surg. Gyn. Obst.*, 110, 734, 1960.

PRUDENTE A. – Cancer dissemination and surgical treatment. *Acta Un. Int. Canc.*, 19, 1104, 1963.

PRUITT J. C., HILBERG A. W., MOREHEAD R. P., MENGOLI H. F. – Quantitative study of malignant cells in local and peripheral of circulating blood. *Surg. Gyn. Obst.*, 114, 179, 1962.

RYALL – Cit. da MUKHTAR F. A. e Coll., 1963.

SMITH R. R., THOMAS L. B., HILBERG H. W. – Cancer cell contamination of operative wounds. *Cancer*, 11, 53, 1958.

SMITH R. R., WESTJ T., MALMGREN R. A., CHU E. W. – Cancer cell wound contamination significance and prophylaxis. *Acta un. Int. Canc.*, 19, 1117, 1963.

ISTITUTO DI SEMEIOTICA CHIRURGICA
DELL'UNIVERSITÀ DI ROMA
Direttore: Prof. G. MARCOZZI

ALTERAZIONI E REAZIONI PRODOTTE DA SOSTANZE LITICHE (acido acetico, acido nitrico) SUI TESSUTI NORMALI

1) Effetti sui fasci vascolo-nervosi

Annali Ital. Di Chirurgia 42:, 1968

E. FANINI

PREMESSA

Particolare interesse, per i chirurgi e per gli oncologi, riveste il problema della disseminazione delle cellule tumorali durante l'intervento chirurgico, la loro immissione nel torrente ematico o linfatico e la loro dispersione nel campo operatorio stesso.

Numerosi lavori segnalano la presenza di cellule tumorali nel sangue di paz. sottoposti ad intervento chirurgico per neoplasia. J. F. POTTER e Coll. (1960) ne hanno trovato ad esempio nel 29% dei casi; J. C. PRUITT (1962) invece nel 30% dei casi. Le percentuali aumentano quando si esamina il sangue delle vene che drenano il tumore, giungendo sino al 43% secondo J. F. POTTER, specialmente quando il prelievo si esegue durante le manovre operatorie (M. DARGENT e Coll. 1963, A. PRUDENTE 1963). È da rilevare che, se la presenza delle cellule tumorali nel sangue non sembra avere molta importanza agli effetti prognostici per H. C. ENGELL (1955), M. DARGENT e Coll. (1963), V. PASQUALUCCI e Coll. (1964), essa sarebbe invece importantissima ai fini delle eventuali metastasi per D. R. FISHER e R. B. TUNBULL (1955), G. E. MOORE (1960), A. PRUDENTE (1963).

Sin dal lontano 1867 C. H. MOORE aveva richiamato l'attenzione sulla riproduzione del tumore sulla ferita operatoria; in seguito anche RYALL (1907), W. H. COLE e Coll. (1954), E. A. MC GREW e Coll. (1954), R. R. SMITH e Coll. (1958), A. PERACCHIA e E. FASANI (1960) ed altri avevano messo in evidenza i danni provocati dalla contaminazione ed avevano consigliato delle norme per evitarla. Esperienze condotte da R. R. SMITH e Coll. nel 1953 e poi ripetute dopo 8 anni, hanno permesso di rilevare che l'acqua di lavaggio del campo operatorio contiene cellule tumorali nel 25,9%. Anche il drenaggio delle ferite ha dimostrato la presenza di cellule tumorali sino a 72 ore dopo l'intervento.

Sommando i casi positivi dopo lavaggio e quelli dopo drenaggio la percentuale aumenta ulteriormente di un terzo (R. R. SMITH 1963).

Anche il tipo istologico avrebbe una discreta importanza nel determinismo delle recidive in loco. Secondo R. R. SMITH infatti le recidive dei cancri mucosi superano in breve tempo il 40% e raggiungono, dopo 5 anni, anche il 53%.

Diversi artifici di tecnica sono stati suggeriti per neutralizzare le disseminazioni di cellule neoplastiche durante l'intervento chirurgico, che possono portare in secondo tempo alle recidive in loco o alle metastasi: tra questi artifizi rientrano i lavaggi del campo operatorio dopo l'intervento. Le sostanze usate finora (soluzioni saline, alcool, etere, formaldeide ecc.) non hanno però dato i risultati sperati.

Si è pensato pertanto di usare, durante l'atto operatorio, alcuni acidi forti a diluizione opportuna, per applicazione topica locale, nel tentativo di distruggere cellule tumorali libere senza arrecare danni eccessivi ai tessuti sani.

Prima di saggiare tali sostanze caustiche sui tessuti neoplastici, abbiamo tuttavia voluto controllare come le varie soluzioni degli acidi in esame agissero sui tessuti normali ed in particolare sui fasci vasculo-nervosi.

CONSIDERAZIONI GENERALI

L'uso clinico di sostanze chimiche per lavare il campo operatorio è probabilmente vecchio come la chirurgia: molti chirurgi usavano sistematicamente lavare le ferite con varie soluzioni. Le sostanze che sono state più usate sono la soluzione salina, l'alcool, l'etere, la formaldeide al 0,5%, la formaldeide con acido picrico (R. R. SMITH 1963); gli antimitotici (W. H. COLE e Coll., 1954); la mostarda azotata (A. PRUDENTE 1963); il sodio ipoclorito (F. A. MUKHTAR e Coll., 1963): i risultati tuttavia sono molto contraddittori. È stato peraltro rilevato da R. R. SMITH e Coll. che, nei casi in cui la ricerca delle cellule tumorali nell'acqua di lavaggio del campo operatorio risultava negativa, l'uso della chemioterapia locale portava ad una netta diminuzione delle recidive. Al contrario, nei casi, in cui l'acqua di lavaggio presentava cellule neoplastiche, la percentuale delle recidive risul-

tava alta, anche dopo l'impiego della chemioterapia per uso topico sul campo operatorio.

Recentemente le ricerche sulle sostanze capaci di distruggere le cellule tumorali hanno conosciuto un nuovo incremento: J. C. MARUZZELLA e D. I. BRENNER (1963) hanno saggiato 50 oli minerali; F. A. MUKHTAR e Coll. (1963) hanno sperimentato a loro volta 60 prodotti fra i quali 9 coloranti, 8 agenti ossidanti, 6 derivati della piridina, 3 antibiotici (mitomicina, actinomicina, spiromicina), 5 antimetaboliti, 3 composti triazinici, 8 agenti alchilanti, 7 chilanti carbammati ed 11 preparati diversi (idrazina, porfirina ecc.). Dopo aver scelto poche sostanze che in vitro avevano dato risultati incoraggianti, questi AA. hanno sperimentato per applicazione locale la mostarda azotata ed il sodio ipoclorito: purtroppo le recidive sono state maggiori dopo l'uso di tali sostanze. Migliori risultati sono stati fortunatamente segnalati da W. H. COLE e Coll. (1951) che hanno irrigato il campo operatorio con antimitotici.

SPERIMENTAZIONE PERSONALE

a) Impostazione

Scopo delle nostre ricerche era di saggiare alcuni prodotti chimici, facilmente repertabili in commercio, che potessero distruggere eventuali frammenti di tessuto tumorale rimasti e repertabili sul campo operatorio durante l'intervento.

Sono stati presi in considerazione, a scopo sperimentale, due acidi forti: l'acido acetico glaciale e l'acido nitrico a varie concentrazioni, acidi di cui conosciamo bene l'effetto sulle sostanze proteiche. Per stabilire quale fosse la concentrazione più idonea a procurare una coagulazione superficiale delle sostanze proteiche senza arrecare danno ai tessuti sottostanti, si è pensato di saggiare le soluzioni in esame su tessuti molto delicati. Sono state pertanto praticate, con le suddette soluzioni, delle spennellature sui fasci vasculo-nervosi siti alle radici degli arti superiori ed inferiori di conigli di media taglia, in modo che, se fossero comparsi degli effetti nocivi, sarebbero stati ben visibili e documentabili.

b) Materiale e metodo

Sono stati impiegati per il complesso delle nostre ricerche 20 conigli di medio peso, suddivisi in 5 gruppi, oltre ad una serie di 3 conigli di controllo.

Dell'acido acetico glaciale è stata saggiata una soluzione Normaldecima (N/10), una soluzione Normale (N), una soluzione al 25% ed una al 50%.

Dell'acido nitrico è stata impiegata una soluzione Normale (N) ed una 1/10 della normale (N/10) o Normaldecima.

In ogni animale la soluzione in esame è stata portata a contatto mediante spennellature con tutti e quattro i fasci vasculo-nervosi e con i tessuti limitrofi, dopo averli scoperti in anestesia locale. Le ferite cutanee sono state poi accuratamente suturate, il tutto ben inteso in campo sterile, e nessun altro trattamento è stato aggiunto. I conigli sono stati riportati nelle loro gabbie e tenuti ad alimentazione normale insieme ai controlli non trattati.

I 4 conigli di ogni gruppo sono stati sacrificati dai 30 ai 35 giorni dopo l'intervento.

c) Osservazioni macro microscopiche

Durante il periodo di osservazione post-operatorio non è stato notato nei conigli alcun disturbo del comportamento, mentre normali sono apparsi la motilità, il riposo, l'appetito e la digestione ed è proseguito normalmente l'accrescimento ponderale. Nessuno dei conigli sottoposti ad intervento è deceduto e tutte le ferite sono normalmente guarite per prima. Dopo aver sacrificato gli animali, sono stati prelevati in blocco le regioni ascellari ed inguinali comprendenti i tessuti cutaneo, sottocutaneo, muscolare ed i fasci vasculo-nervosi.

Nelle zone che erano venute a contatto con l'acido in esame, erano presenti aderenze tra cute, sottocute ed i piani muscolo-aponevrotici; si notava la presenza di un tessuto biancastro, quasi gelatinoso per intenso edema, scarsamente consistente, molto più abbondante nei conigli trattati con le soluzioni più concentrate: tale alterazione si estendeva su tutta la zona trattata con la soluzione. I fasci vasculo-nervosi erano facilmente riconoscibili anche se coperti dall'edema, che era notevole nei casi nei quali era stato impiegato acido al 25 e 50% ed acido nitrico nor-

male. Gli elementi aderivano tra loro lassamente ed era possibile dividerli l'uno dall'altro; non vi era trombosi apparente dei vasi. Nei casi trattati con acidi a più alta concentrazione e specialmente con acido nitrico si aveva invece l'impressione che le pareti fossero ispessite per la maggior consistenza al tatto.

Dai tessuti prelevati sono stati allestiti vari preparati istologici, la osservazione dei quali ha permesso alcune interessanti osservazioni, qui di seguito riferiti:

I Gruppo: Conigli trattati con sol. N/10 di acido acetico.

Il tessuto muscolare striato presenta alla periferia fenomeni degenerativi di vario grado: gli elementi miocellulari in alcune zone tendono a rigonfiarsi ed a perdere la propria tingibilità; in altre zone invece presentano quadri più avanzati in cui il citoplasma si opaca ed assume aspetto granuloso, si osservano anche fenomeni di lisi cellulare con qualche quadro estremo di necrosi cellulare (fig. 1). In mezzo a questo contesto muscolare è ben riconoscibile il fascio vascolo-nervoso. I vasi arteriosi presentano un modico ispessimento dell'avventizia (fig. 2); le vene mostrano un lieve stato di edema subintimale (fig. 3). Medesimo stato edematoso con diastasi delle fibre nervose è reperibile nei tronchi nervosi (fig. 4). Il connettivo grassoso perivascolare è indenne.

II Gruppo: Conigli trattati con sol. N di acido acetico.

Il tessuto muscolare striato presenta le alterazioni sopradescritte lievemente più marcate, sempre però limitate alle zone periferiche. L'arteria presenta un modico ispessimento dell'avventizia. I tronchi nervosi, mentre appaiono indenni nella parte centrale, superficialmente presentano fenomeni di edema e di rigonfiamento delle fibre nervose, le quali spesso appaiono, sulle sezioni trasversali, come dei dischetti otticamente vuoti giacenti in mezzo ad aree fortemente edematose. (figg. 5-6-7-8-9).

III Gruppo: Conigli trattati con sol. al 25% di acido acetico.

I frammenti sono costituiti prevalentemente da tessuto connettivo fibroso. I fasci miocellulari mostrano più marcati i fenomeni regressivi che giungono sino alla lisi cellulare ed alla sostituzione con elementi fibrocitari. I vasi arteriosi e venosi appaiono ispessiti specie nella zona intimale e subintimale. Ma la componente che presenta i fenomeni regressivi più marcati è quella nervosa, i cui fasci sono diastasati per edema. Le singole fibrille ne risultano rigonfie, con aspetto palloniforme sulle sezioni trasverse, e con grado variabile che può arrivare sino alla lisi. Questi fenomeni degenerativi non interessano tutto il nervo ma i gruppi di fibre disposte alla periferia (figg. 10-11-12-13-14).

IV Gruppo: Conigli trattati con sol. al 50% di acido acetico.

Le alterazioni sono ancora più accentuate. Gli elementi miocellulari subiscono profondi fenomeni regressivi, i quali, attraverso fasi di opacamento ed

impastamento citoplasmatico, giungono sino alla lisi cellulare completa. Aumenta lo stroma fibroso che si sostituisce agli elementi muscolari, creando un fitto graticcio in mezzo al quale restano inglobati i residui fascetti miocellulari ancora conservati. I vasi appaiono con parete ispessita per fibrosi ed ispessimento della zona avventiziale e periavventiziale. Anche in questi preparati gli elementi che sono situati lontano dal contatto con la soluzione di acido in esame, rimangono normali o quasi (figg. 15-16-17-18).

V Gruppo: Conigli con sol. N/10 di acido nitrico.

I fasci miocellulari presentano fenomeni regressivi consistenti in rigonfiamento, opacamento, omogeneizzazione delle singole fibrocellule. I vasi si presentano talora ispessiti, ma più spesso con focolai necrobiotici intramurali. Modici i fenomeni di fibrosi periavventiziale. Anche i fascetti nervosi presentano fenomeni regressivi dovuti principalmente allo stato edematoso ed alla dissociazione delle fibre (figg. 19-20-21-22-23).

VI Gruppo: Conigli trattati con sol. N di acido nitrico.

Tutti i vari elementi tissurali sono compromessi in senso degenerativo. I tronchi nervosi si presentano con fibre fortemente dissociate per edema, rigonfie, talora con fenomeni neurolitici diffusi. Su sezioni trasversali le singole fibrille sono ridotte a piccoli anelli tondeggianti ed otticamente vuoti. I vasi presentano la parete rigonfia, omogenea, ispessita e con fibrosi dello strato avventiziale. Anche la componente muscolare striata presenta fenomeni regressivi parcellari sino alla lisi cellulare. Il tessuto muscolare distrutto viene sostituito da tessuto adiposo, od in minor quantità, da tessuto fibroso connettivo neoformato (figg. 24-25-26-27-28-29).

RISULTATI IN SINTESI

Nei 30-35 giorni di osservazione tutti gli animali si sono comportati come il gruppo di conigli lasciato per controllo: i movimenti, l'aspetto, il riposo, la digestione, l'accrescimento ponderale sono stati normali. Nessun coniglio è deceduto prima di essere sacrificato.

Macroscopicamente i tessuti trattati presentavano una certa aderenza tra loro ed una neoformazione di tessuto fibroso in maggiore o minore quantità a seconda della concentrazione della soluzione usata.

Istologicamente le differenze erano notevoli.

a) acido acetico

I tessuti più sensibili alle soluzioni meno concentrate come la Normaldecima (N/10), sono stati quelli muscolari: gli

elementi miocellulari si rigonfiavano, perdevano la tingibilità, o presentavano aspetto granuloso sino alla lisi cellulare. Ad una concentrazione maggiore, sol. Normale (N), anche le fibre nervose, nel tratto periferico, mostravano fenomeni di edema e rigonfiamento. A soluzioni più concentrate i danni erano maggiori con lisi cellulare sia delle fibre muscolari sia delle fibre nervose. Invece i vasi sia venosi che arteriosi presentavano scarsi segni di reazione con ispessimento dell'avventizia e dei tessuti periavventiziali.

b) acido nitrico

Danni decisamente più gravi sono stati riscontrati nei due gruppi di conigli in cui sono state usate le soluzioni Normaldecima (N/10) e Normale (N). Sono state rilevate alterazioni degenerative sino alla necrosi delle miocellule; nelle fibre nervose edema, diastasi delle fibre e necrosi; a carico delle arterie e delle vene è stato riscontrato ispessimento nella zona intimale e subintimale, ed a volte piccole aree di necrobiosi.

CONCLUSIONI

Le soluzioni di acido nitrico, anche molto diluite, arrecano danni non solo alle fibre muscolari ma anche alle fibre nervose, mentre le soluzioni N e N/10 dell'acido acetico risparmiano i vasi ed i nervi, portando alterazioni irreversibili solo negli elementi muscolari. Questa constatazione lascia presupporre che le eventuali cellule tumorali, disseminate nel campo operatorio durante l'intervento chirurgico, essendo molto vulnerabili per la accelerata mitosi, potrebbero essere facilmente distrutte se venissero a contatto delle sopradette soluzioni. Pertanto, visto la buona resistenza dei fasci vasculo-nervosi, si è del parere di poter usare le spennellature con una soluzione N o N/10 di acido acetico sul campo operatorio, dopo l'esportazione del tumore. Per esempio, dopo mastectomia radicale, l'uso di tali spennellature potrebbero distruggere gli eventuali tessuti tumorali che possono rimanere disseminati sul campo operatorio e che spesso sono causa di recidive.

L'esperienza clinica ulteriore ci potrà dire se questi presupposti sperimentali potranno dare una qualche utilità.

Microfotografie dei preparati istologici dei reperti ottenuti dai conigli trattati con Acido acetico N/10 1° Gruppo.

Fig. 1. Frammento di muscolo striato: rigonfiamento e degenerazione granulosa delle fibre. Qualche fenomeno di spezzettamento delle fibre stesse.

Fig. 2. - Arteria: fibrosi dell'avventizia

1° Gruppo: Conigli trattati con acido acetico sol. N/10.

Fig. 3. – Vena periferica: qualche fenomeno di rigonfiamento intimale con protrusione nel lume.

Fig. 4. – Diramazione nervosa periferica: edema fascicolare.

II° Gruppo: Conigli trattati con sol. N di acido acetico.

Fig. 5. – Frammenti di fibre muscolari: dissociazione interstiziale per edema e degenerazione torbida delle fibre, soprattutto di quelle periferiche.

Fig. 6. – Formazioni vascolari nel tessuto cellulo-adiposo. Fibrosi avventiziale a carico delle arterie e delle vene.

II° Gruppo: Conigli trattati con sol. N di acido acetico.

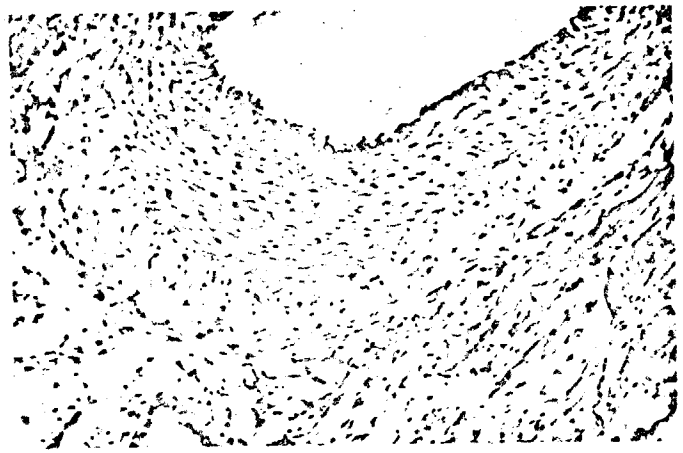


Fig. 7. Particolare del precedente: fibrosi avventiziale peri-arteriosa.

Fig. 8. - Diramazione nervosa periferica: edema e rigonfiamento delle fibre perifericamente disposte nel fascio nervoso, osservati su sezione longitudinale.

II° Gruppo: Conigli trattati con sol. N di acido acetico.

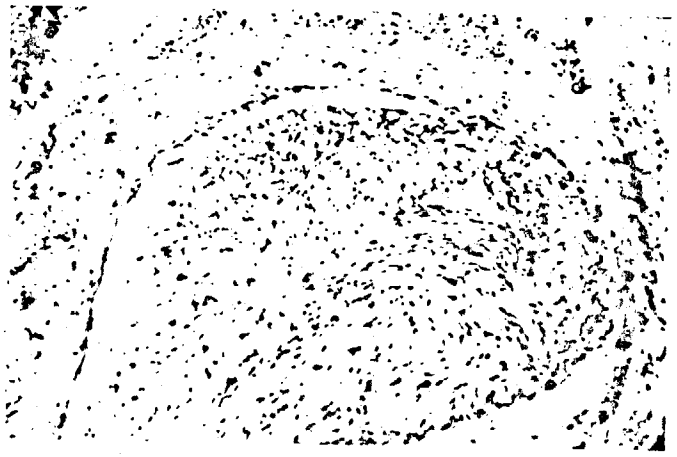


Fig. 9. - Lo stesso quadro osservato su sezione trasversa del tronco nervoso.

III° Gruppo: Conigli trattati con sol. al 25% di acido acetico.

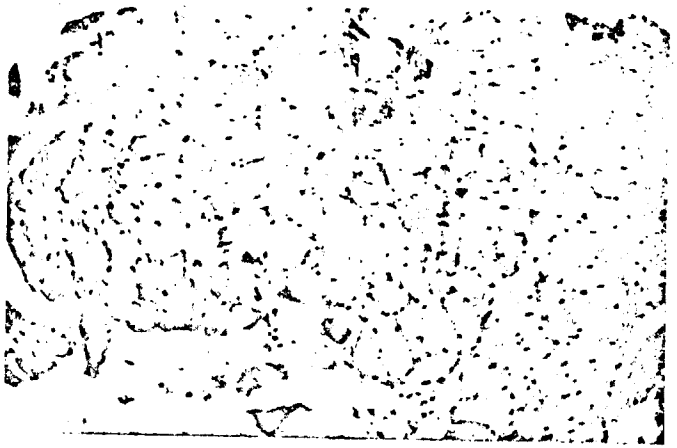


Fig. 10. - Frammenti di fibre muscolari: degenerazione torbida granulosa e sostituzione fibrosa.

III° Gruppo: Conigli trattati con sol. al 25% di acido acetico.

Fig. 11. – Diramazioni nervose: edema dissociativo marcato.

Fig. 12. – Particolare del precedente: degenerazione palloniforme delle fibre nervose e lisi.

III° Gruppo: Conigli trattati con sol. al 25% di acido acetico.

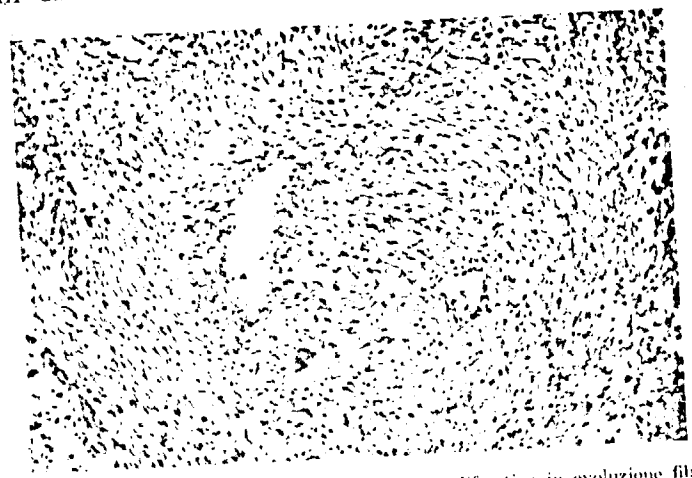


Fig. 13. – Arteria periferica: endo e periarterite proliferativa in evoluzione fibrosa con notevole riduzione del lume vascolare.

Fig. 14. – Vena periferica: periflebite fibrosa.

IVo Gruppo: Conigli trattati con sol. al 50% di acido acetico.

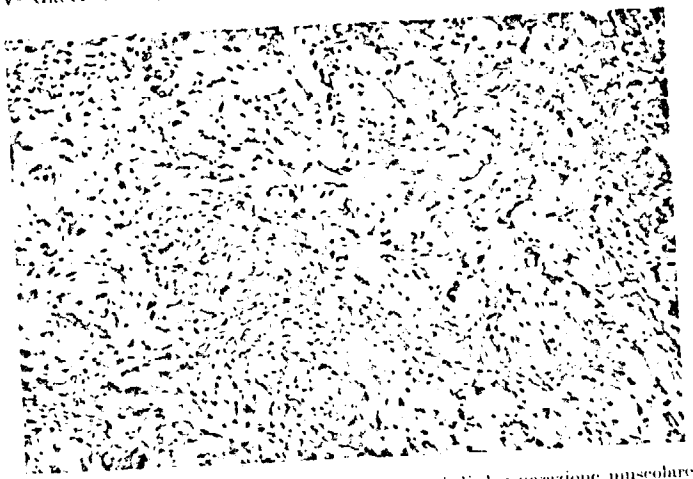


Fig. 15. – Frammenti di fibre muscolari: gravi fatti di degenerazione muscolare con sostituzione fibrosa marcata

Fig. 16. – Arteria periferica: notevole fibrosi avventiziale e periavventiziale.

IVo Gruppo: Conigli trattati con sol. al 50% di acido acetico.

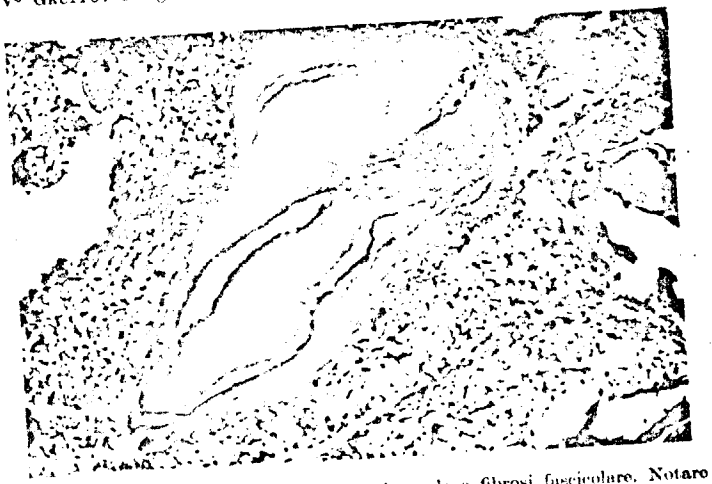


Fig. 17. – Diramazioni nervose: edema perineurale e fibrosi fascicolare. Notare la sclerosi interstiziale che coinvolge il tessuto cellulo-adiposo.

Fig. 18. – Particolare del precedente che dimostra l'edema perineurale e la fibrosi fascicolare del nervo periferico.

V° Gruppo: Conigli trattati con sol. N/10 di acido nitrico.

Fig. 19. – Arteria periferica: ispessimento della media e fibrosi avventiziale.

Fig. 20. – Arteria in sezione longitudinale ad un punto di suddivisione: fibrosi della media e fibrosi avventiziale.

V° Gruppo: Conigli trattati con sol. N/10 di acido nitrico.

Fig. 21. – Dettaglio del precedente.

Fig. 22. – Parete venosa: periflebite sclerotica.

V° Gruppo: Conigli trattati con sol. N/10 di acido nitrico.

Fig. 23. Diramazioni nervose periferiche: edema dissociativo delle fibre, soprattutto di quelle perifericamente disposte.

VI° Gruppo: Conigli trattati con sol. N di acido nitrico.

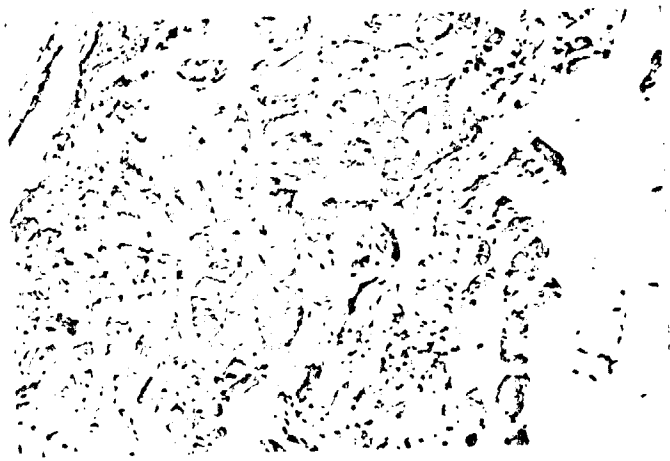


Fig. 24. Frammenti di fibre muscolari: edema dissociativo accentuato con degenerazione torbida sino alla lisi delle fibre.

VI° Gruppo: Conigli trattati con sol. N di acido nitrico.

Fig. 25. – Arteria periferica: modico ispessimento della media.

Fig. 26. – Particolare del precedente: dimostra fenomeni regressivi parcellari della parete.

VI° Gruppo: Conigli trattati con sol. N di acido nitrico.

Fig. 27. – Altra diramazione arteriosa: fibrosi della media e fibrosi avventiziale.

Fig. 28. – Diramazioni nervose: edema perineurale ed interfascicolare con fatti di neurolisi.

VI° Gruppo: Conigli trattati con sol. N di acido nitrico.

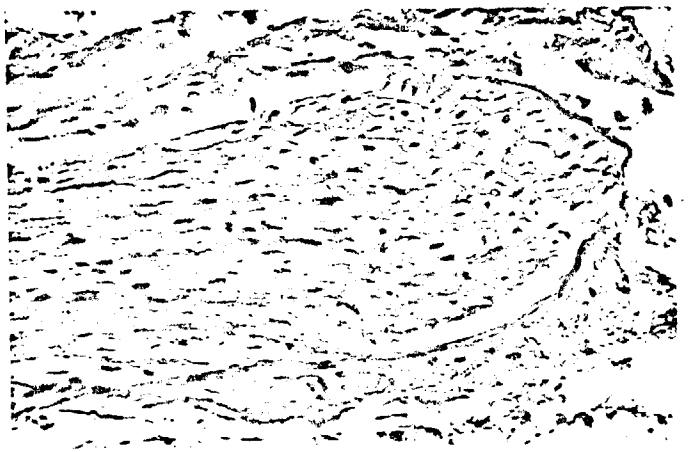


Fig. 29. – Particolare che dimostra l'edema perineurale, la degenerazione palloniforme di alcune fibre a disposizione periferica del tronco nervoso.

RIASSUNTO

L'A. richiama l'attenzione sulla disseminazione delle cellule tumorali durante gli interventi operatori e prospetta l'ipotesi di poter distruggere tali cellule con l'irrorazione del campo operatorio con opportuni medicamenti.

Ha praticato con varie soluzioni di acido acetico ed acido nitrico delle pennellature sui fasci vascolo nervosi degli arti di 20 conigli, con lo scopo di stabilire quale fosse la concentrazione più idonea a procurare una alterazione irreversibile delle cellule superficiali, senza arrecare danno agli elementi vasculo-nervosi. Le soluzioni N e N/10 di acido acetico rispondono allo scopo.

Queste esperienze potrebbero indicarci che alcune sostanze litiche, quali le soluzioni di acido acetico, potrebbero venire usate per applicazione topica locale su tessuti neoplastici rimasti disseminati ed evidenti sul campo operatorio, dopo interventi di exeresi del tumore primitivo.

BIBLIOGRAFIA

Cole W. H., Packard D., Southwick H. W. – Carcinoma of the colon in relation to recurrence. *J.A.M.A.*, 155, 1549, 1954.

Dargent M., Faucon M., Conti C., Pommatan E., Mayer M. – Problème des cellules cancereuses dans le sang circulant. *Acta Un. Int. Canc.*, 19, 1095, 1963.

ENGELL H. C. – Cancer cells in the circulating blood. A clinical study on the occurrence of cancer cells in the peripheral blood and in venous blood draining the tumor area at operation. *Acta Chir. Scandin.*, Suppl. 201, 1955.

FISHER D. R., TURNBULL R. B. The cytologic demonstration and significance of tumor cells in the mesenteric venous blood in patients with colorectal carcinoma. *Surg. Gynec. Obst.*, 100, 102, 1955.

MARUZZELLA J. C., BRENNER D. I. – Cit. da SMITH R. R. e Coll. 1963.

MC GREW E. A., LAWS J. F., COLE W. H. – Free malignant cells in relation to recurrence of carcinoma of colon. *J.A.M.A.*, 154, 1251, 1954.

MOORE C. H. – Cit. da MUKHTAR F. A. e Coll., 1963.

MOORE G. E. – The significance of cancer cells in the blood *Surg. Gyn. Obst.*, 110, 360, 1960.

MUKHTAR F. A., HOFFMEISTER F. S., DI PAOLO J., BROSS I., SHEEHEP W. H., MOORE G. E. – Studies on topical chemioterapy of tumor contaminated surgical wound. *Am. J. Surg.*, 106, 768, 1963.

PERACCHIA A., FESANI F. – Chemioterapia del carcinoma della mammella con trietilentiofosfaramide. *Tumori*, 46, 205, 1960.

PASQUALUCCI V., DESANCTIS C., TRISTAINO B. – Significato e limiti della ricerca di cellule tumorali nel sangue periferico. *Ann. It. Chir.*, 41, 722, 1964.

POTTER J. F., LONGENBAUGH G., CHU E., DILLON J., ROMSDAHL M., MALMGREN A. – The relationship of tumor type and resectability to the incidence of cancer cells in blood. *Surg. Gyn. Obst.*, 110, 734, 1960.

PRUDENTE A. – Cancer dissemination and surgical treatment. *Acta Un. Int. Canc.*, 19, 1104, 1963.

PRUITT J. C., HILBERG A. W., MORLEHEAD R. P., MENGOLI H. F. – Quantitative study of malignant cells in local and peripheral of circulating blood. *Surg. Gyn. Obst.*, 114, 179, 1962.

RYALL – Cit. da MUKHTAR F. A. e Coll., 1963.

SMITH R. R., THOMAS L. B., HILBERG H. W. – Cancer cell contamination of operative wounds. *Cancer*, 11, 53, 1958.

SMITH R. R., WESTJ T., MALMGREN R. A., CHU E. W. – Cancer cell wound contamination significance and prophylaxis. *Acta un. Int. Canc.*, 19, 1117, 1963.

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THE UNIVERSITY OF GENEVA

Research on the Synthesis of Fats from Acetate or Glucose

I. Speed of Incorporation of Acetate in the FAtty Acids of Emaciated Mice.

By P. Favarger and J. Gerlach
Received April 20, 1955
(2 Figures)

Some recent work (1-4) has aroused the suspicion that adipose tissue complets successfully with the liver in the synthesis of fatty acids. The active participation in this synthesis by other tissues, such as the intestines or the lungs, has recently been shown by several authors (5,6). However, none of these works have given definitive proof which would cause the liver to be stripped of its essential role in the synthesis of fatty acids. Experiments done in vitro never give quantitative results, except if a study is done of the same tissue, taken from the same animal, and the effect of some substance added to the milieu under rigorously parallel conditions is observed. This doesn't enable presumptions to be made about the relative participation of two different tissues in the same phenomenon. The measure of the specific activities in the different organs, after the administration to the animal of a marked precursor doesn't allow either unequivocal conclusions to be made when the measurements are made after several hours. The newly formed fatty acids are far from being uniformly distributed in the tissues of the cells. An organ of intense metabolism could theoretically send, by way of the blood, to other organs, fatty acids more strongly marked than the average of its own fatty acids. The measure of the specific aggregate activities in each tissue

activities in each tissue would thus give only a very inexact picture of the synthesizing activity of each of them. This problem is particularly important for organs which have multiple distinct activities, such as the liver. Further, several studies (7-9) have shown how the incorporation of acetate into fatty acids may be rapid. It was also probable that the distribution in the various organs differed according to the method of administration. Therefore, we examined the distribution of newly formed fatty acids in mice by killing the animals a few minutes after an intraperitoneal or subcutaneous injection of 1-C^{14} acetate. Our principal aim being to elucidate the role of the adipose tissue in lipogenesis, we studied the incorporation of acetate in emaciated mice during the period of the reconstitution of their fat reserves.

Experimental Section

White mice of both sexes and weighing 22 to 33 g were subjected to a preliminary fast of 48 hours, then were given 1 to 3 g of bread to eat during 5 to 6 hours. They received 2.5 to 5.0 μc of 1-C^{14} acetate (0.1 or 0.2 mg) in an isotonic injection into the peritoneal cavity or under the skin of a rear leg. They were killed 3, 12, or 30 minutes later by decapitation, and the liver, the mesenteric fat, the brown interscapular fat and the carcass were immediately fixed in boiling alcohol. The organs were saponified in 10% alcoholic potash for two hours (6 hours for the carcass). The in-saponifiable parts were eliminated by petroleum ether, then the fatty acids were extracted which were first washed two times by "washing out" with a solution of sodium acetate, then two times with pure water.

The fatty acids are dissolved in alcohol and then a sample is taken of the aliquot parts (1 to 5mg) corresponding to a volume of 0.2 to 0.5cm^3.

They are evaporated directly in aluminum cups having diameters of 22mm, and a brim of 8mm. The surface obtained is satisfactory if the following precautions are taken:

1. The cups must be cleaned with soda, passed through nitric acid and carefully rinsed with water.
2. The solvent must be ethanol.
3. The evaporation must take place under a lamp, the temperature at cup level reaching 50°.

The results are compared with those reported in a reference curve obtained under the same conditions with 1-C^{14} palmitic acid dissolved in the fatty acids of mice. Generally, enough material is prepared so that the measurements can be repeated several times. The agreement is very satisfactory. The measurements are done by means of a "windowless flow counter."

Results and Discussion

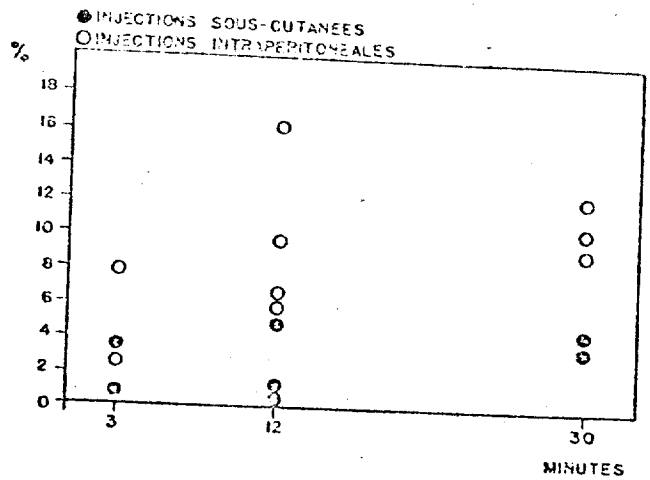
The conditions of nourishment of the mice at the time of injection have a great influence. If bread is given to them after a fast of 48 hours, and they are killed less than a half-hour later (the acetate injection preceding the sacrifice by 3 minutes), 4.4 to 10.2% of the injected substance is found in their fatty acids. If one waits an hour after their meal, the proportion falls to 1 to 2%. If the mice are fasting, they incorporate less then 1% of the injected acetate. Hutchens and his colleagues have gotten analagous results for rats killed four hours after the injection of acetate (10, 11).

If the specific activity of the fatty acids in the injected leg is measured, it is found to be 3 to 7 times stronger than that in the rest of the organism. If the injection is intraperitoneal, the liver and the mesenteric fat are comparatively more active than if the injection is sub-

cutaneous. Thus, after an intraperitoneal injection, the specific activity of the fatty acids of the mesentery is less in only one case out of 13 than the average activity in the whole mouse, and in nine cases, it is two to six times greater. After a subcutaneous injection the activity of the same fats is greater than the average only one time out of seven experiments.

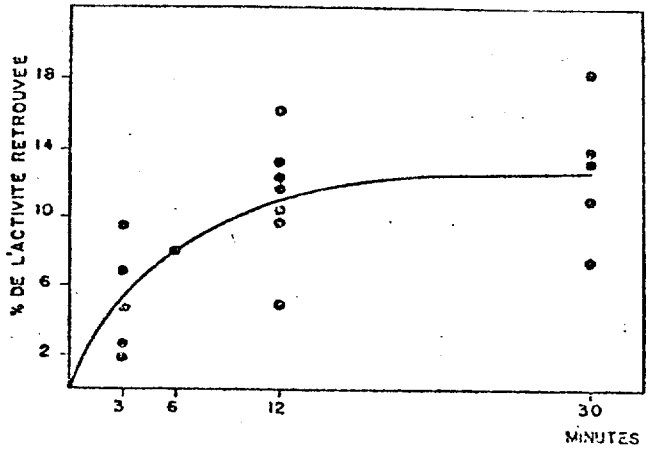
Figure 1 enables the verification that the total activity of the hepatic fatty acids is clearly greater after intraperitoneal injections. Furthermore, the proportion of the marked fatty acids recovered in the liver increases with time. This phenomenon is even more apparent with intravenous injections, and its significance is discussed in another work. (12).

Figure 1 Proportion of newly formed fatty acids present in the liver of the mouse in relation to the newly formed fatty acids in the total mouse.



● Subcutaneous injections
o Intraperitoneal injections

Figure 2 Proportion of 1-C^{14} acetate refound in the form of fatty acids in mice killed 3 to 30 minutes after subcutaneous or intraperitoneal injection.



The considerable influence of the site of injection on the distribution of the specific activity of the fatty acids proves the rapidity of the incorporation. This rapidity stands out in the examination of figure 2 in which the points represent the proportion of the injected substance which is recovered in the form of fatty acids in mice killed 3, 6, 12 or 30 minutes after injection. The variations from animal to animal are considerable, but there are not significant differences between intravenous and intraperitoneal injections relating to the aggregate activity. The variations observed in each group of mice arises, first of all, from the very different states of emaciation, in spite of identical dietary conditions. The most emaciated mice generally had fats of strong specific activity because the newly formed acids are only slightly diluted by the pre-existant fats, but some of the mice were visibly very weakened and didn't incorporate as much acetate as the others.

We aren't giving a table of results in this first article. Actually, this is designed to bring forth a fact of great practical importance, the mis-

understanding of which has tainted with errors many works published in this field. The intraperitoneal or subcutaneous injections are not appropriate to establish comparisons between the lipogenic capacity of the different organs. The incorporation of the C of the acetate into the fatty acids is so rapid that a large proportion of the injected substance doesn't have time to be re-distributed in the whole organism before synthesis. It is therefore necessary to use intravenous injections so that the acetate will reach as quickly as possible the sites of eventual synthesis.

Summary

During the period of the reconstitution of their fat reserves, some mice received intraperitoneal or subcutaneous injections of 1-C^{14} acetate and were killed a few minutes later. The newly formed fatty acids of the carcass and of various tissues were determined. The injection site has a great influence on the distribution of the fatty acids. The speed of incorporation of the acetate in the fatty acids is too great to allow the measurement of the lipogenic capacity of the different tissues by injecting the precursor subcutaneously or intraperitoneally. Intravenous injections must be used.

Bibliography

1. Shapiro B. and Wertheimer E.: The synthesis of fatty acid in adipose tissue in vitro. J. biol. Chem. 173, 725-728 (1948).

2. Feller, D. D.: Metabolism of adipose tissue. I. Incorporation of acetate into lipids by slices of adipose tissue. J. biol. Chem. 206, 171-180 (1954).

3. Hausberger, F. X., Milstein, S. W. and Rutman, R.J.: The influence of insulin on glucose utilization in adipose and hepatic tissues in vitro. J. biol. Chem. 208, 431-438 (1954).

4. Fararger, P. and J. Gerlach: Research on the synthesis of fats in the adipose tissue of the rat. Helv. Physiol. Acta 12, C15-C17 (1954).

5. Popjak G. and Beeckmans M. L.: Extrahepatic lipid synthesis. Biochem. J. 47, 233-238 (1950).

6. Bernhard K. and Bullet F.: Die Bildung von Fettsauren im Intestinaltractus. Helv. Chim. Acta 30, 1784-1786 (1947).

7. Hevesy G., Ruyssen R. and Beeckmans M.L.: Turnover rate of the fatty acids of the liver. Experientia 7, 144-146 (1951).

8. Ruyssen, R. and H. Anciaux: The speed of incorporation of C^{14} in fatty acids from acetate. International colloquium on the biochemical problems of lipids, p. 299-310. Brussels. (1953).

9. Van Bruggen J. T., Hutchens T. T., Claycomb C. K., and West E. S.: Time course of lipide labelling in the intact mouse and rat. J. biol. Chem. 200, 31-37 (1953).

10. Hutchens T. T., Van Bruggen J. T. and West E.S.: Fatty acid and cholesterol synthesis rates in the intact rat. Arch. Biochem. Biophysics 52, 261-268 (1954).

11. Hutchens T. T., Van Bruggen J. T., Cockburn R. M. and West E. S.: The effect of fasting upon tissue lipogenesis in the intact rat. J. biol. Chem. 208, 115-122 (1954).

12. Favarger P. and J. Gerlach: Research on the synthesis of fats from acetate or glucose. II. The respective roles of the liver, the adipose tissue and certain other tissues in lipogenesis in mice. Helv. Physiol. Acta 13, 96-105. (1955).

Helv. Physiol. Acta 13, 91-95 (1955)

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Recherches sur la synthèse des graisses à partir d'acétate ou de glucose

I. Vitesse d'incorporation de l'acétate dans les acides gras de la souris amaigrie

Par P. Favarger et J. Gerlach

Reçu le 20 avril 1955

(2 figures)

Un certain nombre de travaux récents [1-4] ont permis de soupçonner le tissu adipeux de concurrencer victorieusement le foie dans la synthèse des acides gras. L'active participation à cette synthèse d'autres tissus comme l'intestin ou le poumon, a été mise en évidence par plusieurs auteurs [5, 6]. Cependant, aucun de ces travaux n'apporte de preuve définitive permettant de détrôner le foie de son rôle essentiel dans la synthèse des acides gras. Les essais in vitro ne peuvent jamais donner de résultats quantitatifs, sauf si pour un même tissu, prélevé sur le même animal, on étudie l'action de telle ou telle substance ajoutée au milieu dans des conditions rigoureusement parallèles. Ils ne permettent d'obtenir que des présomptions sur la participation relative de deux tissus différents à un même phépomène. La mesure des activités spécifiques dans différents organes, après administration à l'animal d'un précurseur marqué, ne permet pas non plus de conclusion univoque lorsque les mesures sont faites après plusieurs heures. Les acides gras néoformés sont loin d'être répartis uniformément dans les tissus ou dans les cellules. Un organe à métabolisme intense pourrait donc théoriquement envoyer par le sang à un autre organe des acides gras plus fortement marqués que la moyenne de ses propres acides gras. La mesure des activités spécifiques globales dans chaque tissu ne donnerait ainsi qu'une image très inexacte de l'activité synthétisante de chacun d'eux. Ce danger est particulièrement à craindre pour les organes qui ont de multiples activités distinctes, comme le foie. En outre, plusieurs travaux [7-9] ont montré combien l'incorporation de l'acétate dans les acides gras pouvait être rapide. Il était aussi vraisemblable que la répartition dans les différents organes différât selon le mode d'administration. Nous avons donc examiné la répartition des acides gras néoformés chez la souris, en tuant les animaux peu de minutes après une injection intrapéritonéale ou sous-cutanée d'acétate 1-C^{14}. Notre but principal étant d'élucider le rôle du tissu adipeux dans la lipogenèse, nous avons étudié l'incorporation d'acétate chez des souris amaigries en période de reconstitution des réserves.

Partie expérimentale

Des souris blanches des deux sexes et pesant 22 à 33 g sont soumises à un jeûne préalable de 48 heures, puis mangent 1 à 3 g de pain pendant 5 à 6 heures. Elles reçoivent 2,5 ou 5,0 μc d'acétate 1-C^{14} (0,1 ou 0,2 mg) en injection isotonique dans la cavité péritonéale ou sous la peau d'une patte postérieure. On les tue 3, 12 ou 30 minutes plus tard par décapitation, et fixe immédiatement le foie, la graisse mésentérique, la graisse brune interscapulaire et la carcasse dans l'alcool bouillant. Les organes sont saponifiés par la potasse alcoolique 10% pendant 2 h. (6 h. pour la carcasse). On élimine l'insaponifiable à l'éther de pétrole, puis extrait les acides gras qui sont lavés d'abord deux fois par «washing out» avec une solution d'acétate de Na, puis deux fois à l'eau pure.

Les acides gras sont dissous dans l'alcool et l'on en prélève des parties aliquotes (1 à 5 mg) correspondant à un volume de 0,2 à 0,5 cm^3. On évapore directement dans des cupules d'aluminium de 22 mm de diamètre, possédant un rebord de 8 mm. La surface obtenue est satisfaisante si l'on prend les précautions suivantes:

1. Les cupules doivent être décapées à la soude, passées à l'acide nitrique et rincées soigneusement à l'eau.
2. Le dissolvant doit être de l'éthanol.
3. L'évaporation doit se faire sous une lampe, la température au niveau de la cupule atteignant 50°.

Les résultats sont comparés avec ceux qui sont reportés sur une courbe de référence obtenue dans les mêmes conditions avec de l'acide palmitique 1-C^{14} dissous dans des acides gras de souris. On dispose en général d'assez de matériel pour répéter les mesures plusieurs fois. La concordance est très satisfaisante. Les mesures sont faites au moyen d'un «windowless flow counter».

Résultats et discussion

Les conditions d'alimentation des souris au moment de l'injection ont une grande influence. Si on leur a donné du pain après un jeûne de 48 h. et qu'on les tue moins d'une demi-heure plus tard (l'injection d'acétate précédant de 3 minutes le sacrifice), on retrouve dans leurs acides gras 4,4 à 10,2% de l'activité injectée. Si l'on attend 1 heure après leur repas, la proportion tombe à 1 à 2%. Si les souris sont à jeun, elles incorporent moins de 1% de l'activité injectée. *Hutchens* et ses collaborateurs ont fait des constatations analogues pour des rats tués 4 h. après injection d'acétate [10, 11].

Si l'on mesure l'activité spécifique des acides gras de la patte injectée, on constate qu'elle est 3 à 7 fois plus forte que celle du reste de l'organisme. Si l'injection est intrapéritonéale, le foie et la graisse mésentérique sont comparativement plus actifs que si l'injection est sous-cutanée. Ainsi, après une injection intrapéritonéale, l'activité spécifique des acides gras du mésentère est inférieure dans un seul cas sur 13 à l'activité moyenne de toute la souris, et dans 9 cas, elle est de 2 à 6 fois supérieure. Après une injection sous-cutanée, l'activité de ces mêmes graisses est supérieure à la moyenne une seule fois sur 7 expériences.

La fig. 1 permet de constater que l'activité totale des acides gras hépatiques est nettement plus grande après des injections intrapéritonéales. En outre, la proportion d'acides gras marqués retrouvés dans le foie augmente

avec le temps. Ce phénomène est encore plus apparent pour les injections intraveineuses, et sa signification est discutée dans un autre travail [12].

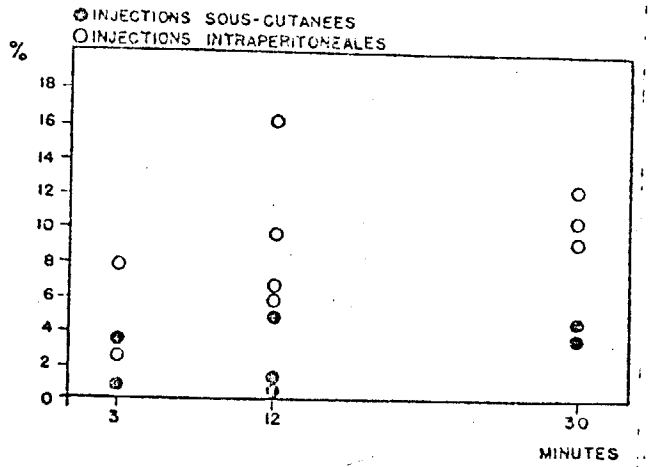
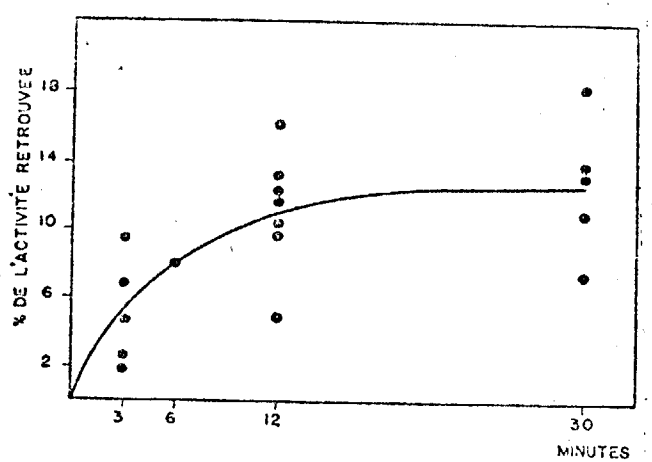


Fig. 1. Proportion des acides gras néoformés présents dans le foie de souris par rapport aux acides gras néoformés de la souris totale.



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Summary

Mice receive, during the reconstitution of their fat reserves, intraperitoneal or subcutaneous injections of 1-C^{14} acetate and are killed a few minutes after. The neo-formed fatty acids of the carcass and of different tissues are determined. The site of injection has a great influence on the repartition of the fatty acids. The speed of acetate incorporation in the fatty acids is too high to allow the determination of lipogenic capacity of the different tissues by injecting the precursor subcutaneously or intraperitoneally. Intravenous injections must be used.

1. *Shapiro B.* and *Wertheimer E.*: The synthesis of fatty acid in adipose tissue in vitro. J. biol. Chem. 173, 725–728 (1948).
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3. *Hausberger, F. X.*, *Milstein, S. W.* and *Rutman, R. J.*: The influence of insulin on glucose utilization in adipose and hepatic tissues in vitro. J. biol. Chem. 208, 431–438 (1954).

4. *Favarger, P.* et *Gerlach J.:* Recherches sur la synthèse des graisses dans le tissu adipeux du rat. Helv. Physiol. Acta **12**, C 15–C 17 (1954).
5. *Popjak G.* and *Beeckmans M. L.:* Extrahepatic lipid synthesis. Biochem. J. **47**, 233–238 (1950).
6. *Bernhard K.* and *Bullet F.:* Die Bildung von Fettsäuren im Intestinaltractus. Helv. Chim. Acta **30**, 1784–1786 (1947).
7. *Hevesy G., Ruyssen R.* and *Beeckmans M. L.:* Turnover rate of the fatty acids of the liver. Experientia **7**, 144–146 (1951).
8. *Ruyssen R.* et *Anciaux H.:* La vitesse d'incorporation du C^{14} dans les acides gras à partir d'acétate. Colloque international sur les problèmes biochimiques des lipides, p. 299–310. Bruxelles 1953.
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10. *Hutchens T. T., Van Bruggen J. T.* and *West E. S.:* Fatty acid and cholesterol synthesis rates in the intact rat. Arch. Biochem. Biophysics **52**, 261–268 (1954).
11. *Hutchens T. T., Van Bruggen J. T., Cockburn R. M.* and *West E. S.:* The effect of fasting upon tissue lipogenesis in the intact rat. J. biol. Chem. **208**, 115–122 (1954).
12. *Favarger P.* et *Gerlach J.:* Recherches sur la synthèse des graisses à partir d'acétate ou de glucose. II. Les rôles respectifs du foie, du tissu adipeux et de certains autres tissus dans la lipogenèse chez la souris. Helv. Physiol. Acta **13**, 96–105 (1955).

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Recherches sur la synthèse des graisses à partir d'acétate ou de glucose

I. Vitesse d'incorporation de l'acétate dans les acides gras de la souris amaigrie

Par P. Favarger et J. Gerlach

Reçu le 20 avril 1955

(2 figures)

Un certain nombre de travaux récents [1–4] ont permis de soupçonner le tissu adipeux de concurrencer victorieusement le foie dans la synthèse des acides gras. L'active participation à cette synthèse d'autres tissus comme l'intestin ou le poumon, a été mise en évidence par plusieurs auteurs [5, 6]. Cependant, aucun de ces travaux n'apporte de preuve définitive permettant de détrôner le foie de son rôle essentiel dans la synthèse des acides gras. Les essais in vitro ne peuvent jamais donner de résultats quantitatifs, sauf si pour un même tissu, prélevé sur le même animal, on étudie l'action de telle ou telle substance ajoutée au milieu dans des conditions rigoureusement parallèles. Ils ne permettent d'obtenir que des présomptions sur la participation relative de deux tissus différents à un même phénomène. La mesure des activités spécifiques dans différents organes, après administration à l'animal d'un précurseur marqué, ne permet pas non plus de conclusion univoque lorsque les mesures sont faites après plusieurs heures. Les acides gras néoformés sont loin d'être répartis uniformément dans les tissus ou dans les cellules. Un organe à métabolisme intense pourrait donc théoriquement envoyer par le sang à un autre organe des acides gras plus fortement marqués que la moyenne de ses propres acides gras. La mesure des activités spécifiques globales dans chaque tissu ne donnerait ainsi qu'une image très inexacte de l'activité synthétisante de chacun d'eux. Ce danger est particulièrement à craindre pour les organes qui ont de multiples activités distinctes, comme le foie. En outre, plusieurs travaux [7–9] ont montré combien l'incorporation de l'acétate dans les acides gras pouvait être rapide. Il était aussi vraisemblable que la répartition dans les différents organes différât selon le mode d'administration. Nous avons donc examiné la répartition des acides gras néoformés chez la souris, en tuant les animaux peu de minutes après une injection intrapéritonéale ou sous-cutanée d'acétate 1-C^{14}. Notre but principal étant d'élucider le rôle du tissu adipeux dans la lipogenèse, nous avons étudié l'incorporation d'acétate chez des souris amaigries en période de reconstitution des réserves.

Partie expérimentale

Des souris blanches des deux sexes et pesant 22 à 33 g sont soumises à un jeûne préalable de 48 heures, puis mangent 1 à 3 g de pain pendant 5 à 6 heures. Elles reçoivent 2,5 ou 5,0 μc d'acétate 1-C^{14} (0,1 ou [illegible] ml) en injection isotonique dans la cavité péritonéale ou sous la peau d'une patte postérieure. On les tue 3, 12 ou 30 minutes plus tard par décapitation, et fixe immédiatement le foie, la graisse mésentérique, la graisse brune interscapulaire et la carcasse dans l'alcool bouillant. Les organes sont saponifiés par la potasse alcoolique 10% pendant 2 h. (6 h. pour la carcasse). On élimine l'insaponifiable à l'éther de pétrole, puis extrait les acides gras qui sont lavés d'abord deux fois par «washing out» avec une solution d'acétate de Na, puis deux fois à l'eau pure.

Les acides gras sont dissous dans l'alcool et l'on en prélève des parties aliquotes (1 à 5 mg) correspondant à un volume de 0,2 à 0,5 cm^3. On évapore directement dans des cupules d'aluminium de 22 mm de diamètre, possédant un rebord de 8 mm. La surface obtenue est satisfaisante si l'on prend les précautions suivantes:

1. Les cupules doivent être décapées à la soude, passées à l'acide nitrique et rincées soigneusement à l'eau.
2. Le dissolvant doit être de l'éthanol.
3. L'évaporation doit se faire sous une lampe, la température au niveau de la cupule atteignant 50°.

Les résultats sont comparés avec ceux qui sont reportés sur une courbe de référence obtenue dans les mêmes conditions avec de l'acide palmitique 1-C^{14} dissous dans des acides gras de souris. On dispose en général d'assez de matériel pour répéter les mesures plusieurs fois. La concordance est très satisfaisante. Les mesures sont faites au moyen d'un «windowless flow counter».

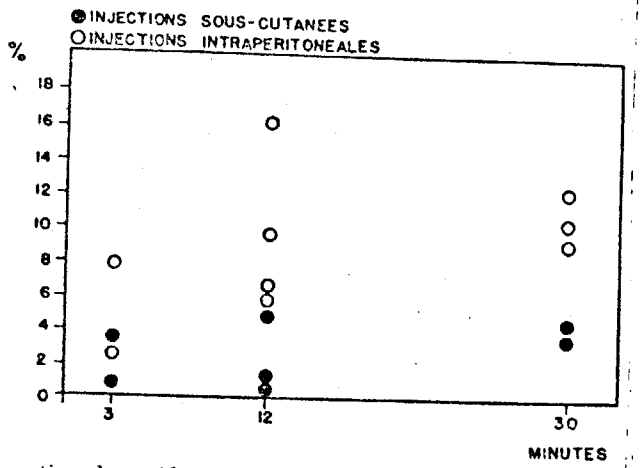
Résultats et discussion

Les conditions d'alimentation des souris au moment de l'injection ont une grande influence. Si on leur a donné du pain après un jeûne de 48 h. et qu'on les tue moins d'une demi-heure plus tard (l'injection d'acétate précédant de 3 minutes le sacrifice), on retrouve dans leurs acides gras 4,4 à 10,2% de l'activité injectée. Si l'on attend 1 heure après leur repas, la proportion tombe à 1 à 2%. Si les souris sont à jeun, elles incorporent moins de 1% de l'activité injectée. *Hutchens* et ses collaborateurs ont fait des constatations analogues pour des rats tués 4 h. après injection d'acétate [10, 11].

Si l'on mesure l'activité spécifique des acides gras de la patte injectée, on constate qu'elle est 3 à 7 fois plus forte que celle du reste de l'organisme. Si l'injection est intrapéritonéale, le foie et la graisse mésentérique sont comparativement plus actifs que si l'injection est sous-cutanée. Ainsi, après une injection intrapéritonéale, l'activité spécifique des acides gras du mésentère est inférieure dans un seul cas sur 13 à l'activité moyenne de toute la souris, et dans 9 cas, elle est de 2 à 6 fois supérieure. Après une injection sous-cutanée, l'activité de ces mêmes graisses est supérieure à la moyenne une seule fois sur 7 expériences.

La fig. 1 permet de constater que l'activité totale des acides gras hépatiques est nettement plus grande après des injections intrapéritonéales. En outre, la proportion d'acides gras marqués retrouvés dans le foie augmente

avec le temps. Ce phénomène est encore plus apparent pour les injections intraveineuses, et sa signification est discutée dans un autre travail [12].



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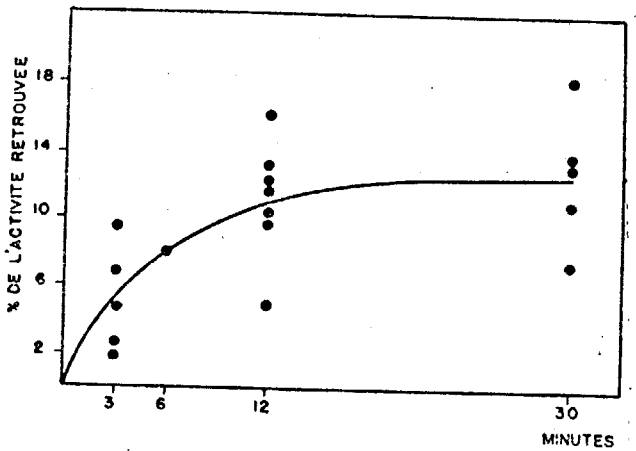

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Russian Translation

Blood Coagulation Changes in Acetic Acid Poisoning

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Source: Klin. Med. (Moskva) 47: 84-87, July 1969.

In cases of acetic acid poisoning, thrombosis and hemorrhages are frequently noted in internal organs (L.I. Serbilat'ev, A.V. Rusakov, M.I. Shevlyagina, G.G. Zakharov, A.F. Rubezhanskii, A.S. Lonshchakova and A.I. Zikeeva, V.K. Derboglav). Formation of hemorrhages is usually linked with a higher vascular permeability as a result of the toxic effect of acetic acid on vascular walls, or is considered as the result of vascular injury due to products of increased protein breakdown formed during burns of tissues in the gastrointestinal tract (M.I. Shevlyagina). The mechanism responsible for the formation of intravascular thromboses of internal organs has even been studied to a lesser extent.

At present, not a single case of vascular thrombosis can be examined without taking into consideration the blood coagulation system. However, we have been unable to locate in the published literature any studies describing the status of blood coagulation processes during acetic acid poisoning. An analysis of the changes taking place in the blood coagulation system during acetic acid poisoning will allow us to get a better idea about certain aspects of the pathogenesis of the disease, to determine the cause of certain complications, and possibly also to develop a more rational therapy.

We have studied the blood coagulation system in 70 patients, ranging in age from 14 to 76 years, suffering from acetic acid poisoning; the patients included 19 men and 51 women. A single analysis of blood coagulation was performed on 47 patients, and a dynamic analysis on 23 patients. Indices of the coagulation pattern (coagulogram) of 50 practically healthy people were used as controls (I.A. Kopteva).

The following indices were determined: general blood coagulation activity (coagulation time according to Lee and White, plasma recalcification time according to Bergerhof and Rock, heparin plasma tolerance according to Poller), first-stage blood coagulation (thromboplastin activity according to Graham, Langdale and Brinkhouse, as modified by V.P. Baluda, antithromboplastin activity according to V.P. Baluda and V.V. Chernaya), second-stage blood coagulation (prothrombin index according to Tugolukov, prothrombin concentration by the two-step method of Weyer and Siegers, as modified by V.P. Baluda, Acglobulin concentration according to Lery and Weyer, proconvertin concentration according to Ovren and Haas, antithrombin activity according to Del Buano and Pasero), third-stage blood coagulation (fibrinogen concentration by the dry air method, activity of the fibrin-stabilizing factor according to V.P. Baluda, N.A. Zhukova, Zh.I. Rukazen'kova, fibrinogen B), and the fibrinolytic activity according to Koval'skii, Kopek and Niverovskii.

Our studies showed that, in acetic acid poisoning, significant changes occur in the functional state of the blood coagulation system, the nature of which is determined to a considerable extent by the time lapse since the moment of poisoning. For this reason, all patients were divided into 2 groups: the first one included 30 patients, in which blood coagulation

was investigated basically during the first 24 hours after poisoning; the second group comprised 40 patients, examined 2-3 days after poisoning.

In all patients of the first group, an increase of the general blood coagulation activity was noted, as demonstrated primarily by the statistically reliable increase of the heparin plasma tolerance, averaging 3.2 min. ($p<0.001$; normal 4.3 min.). The shorter time of blood coagulation, averaging 6.5 min. ($p<0.5$; normal 7.1 min.), and the shorter recalcification time, averaging 94 sec ($p<0.001$; normal 109 sec), also indicate the greater coagulability of blood in this group of patients. Clinically, this hypercoagulemia manifests itself in the fact that, during venipuncture, blood often coagulates in the syringe or even in the needle. We have observed this fact even in those cases when blood was taken with silicone-treated needles and syringes.

After examining the other indices of the coagulogram, we came to the conclusion that the increase of the general blood coagulation activity is due primarily to changes (shifts) in the first phase of hemostasis. Among the 26 examined patients in this group, only 5 had a normal thromboplastin activity, while in the remaining patients this activity was increased on an average up to 65.7%, the normal value being 47.7% ($p<0.001$). It should be noted that the increased thromboplastin activity was frequently accompanied by a decrease of the antithromboplastin activity, which was observed in 17 of the 26 examined patients.

The antithrombin activity of the blood was lowered in half of the patients examined. The concentration of procoagulants in the second stage of blood coagulation (prothrombin index, Ac-globulin, proconvertin and prothrombin) was close to normal in light poisoning cases and was

somewhat lower in more severe cases. Inhibition of the anticoagulant activity of blood is no doubt one of the factors causing the onset of blood hypercoagulation.

It is interesting to note that the increased blood coagulability in the patients was combined with an inhibition of fibrinolysis. Out of the 30 patients in this group, a significant inhibition of fibrinolysis was noted in 28 patients (from 5 to 15 hours, the normal value being 3-5 hours). During hypercoagulemia, inhibition of fibrinolysis is an unfavorable factor. In addition, the activity of the fibrin-stabilizing factor exhibited a 1.5-2-fold increase in the great majority of patients. An average increase in fibrinogen concentration of up to 485 mg % ($p<0.001$; normal value 402 mg%) was noted.

A characteristic feature in patients of the second group was the lowered blood coagulability, as indicated by the statistically reliable decrease in the heparin blood tolerance, averaging 6 min ($p<0.001$), and the longer plasma recalcification time, up to 132 sec ($p<0.001$), and longer blood coagulation time, up to 9 min. ($p<0.001$).

The lowered general coagulating activity of the blood was accompanied in 2/3 of the patients by a decrease of the thromboplastin activity. In individual patients, the latter was equal to zero, while in others it was close to normal or higher. However, in spite of the increase of this index, the blood coagulability, as the final result of the blood coagulation process, was reduced, since in almost all patients of the second group a considerable increase of the antithromboplastin and antithrombin activity was noted; the former averaged 177% ($p<0.001$), while the latter averaged 147% ($p<0.01$). The concentration of procoagulants in the second stage of

blood coagulation decreased in cases of severe poisoning.

The fibrinogen concentration was higher than in patients of the first group, averaging 566 mg % ($p<0.001$). In the majority of the patients examined (32 out of 37) fibrinolysis was still considerably inhibited. The activity of the fibrin-stabilizing factor remained at its previous level, i.e. it was increased.

In acetic acid poisoning, the blood coagulation system undergoes phase changes. Shortly after poisoning, an increase in the blood coagulability was noted. The state of accelerated coagulation was due primarily to the increased thromboplastin and to the inhibited anticoagulant activity. At the same time, hypercoagulation combined with inhibition of fibrinolysis, hyperfibrinogenemia and the more intense activity of the fibrin-stabilizing factor create the prerequisites for the development of intravascular thrombosis. A further confirmation of this fact is the positive fibrinogen B reaction, noted in 10 patients.

The hypocoagulation phase, which sets in after the period of increased blood coagulation, apparently constitutes the response reaction of the organism to the threat of intravascular thrombosis formation.

The slowing down of blood coagulation processes remains in force in most patients even at a later date (1-2 weeks later), and this fact probably contributes to the appearance of late gastric hemorrhages.

In conclusion, it should be noted that the rate of expression of disorders in the blood coagulation system is correlated to a considerable extent with the severity of injuries sustained.

Bibliography

1. Baluda V.P., Malyarovskii V.N., Oyvin I.A. Laboratornye metody issledovaniya svertyvayushchey sistemy krovi (Laboratory Methods for the Study of the Blood Coagulation System), Moscow, 1962.

2. Derboglav V.K. In book: Sudebno-meditsinskaya ekspertiza i kriminalistika na sluzhbe sledstviya (Medico-legal testimony and criminal science at the service of legal inquest), Grozngy, 1962, issue 3, p. 285.

3. Zakharov G.G. In book: Sbornik nauchnykh rabot Minsk. med. in-ta (Collection of scientific works of the Minsk Medical Institute), Minsk, 1952, vol. 18, p. 199.

4. Kopteva I.A. In book: Skhema issledovaniya bol'nogo v terapevticheskoy klinike (Method of examination of patients in a therapeutic clinic), Kuybyshev, 1967, p. 19.

5. Lonshchakova A.S., Zikeeva A.I. In book: Problema zameshcheniya krovi retsipienta krovyu donora (The problem of substitution of recipient blood by donor blood), Alma-Ata, 1958, p. 158.

6. Rubezhanskii A.F. Tezisy dokl. Nauchnoi sessii Kubansk. med. in-ta (Summary of reports of the scientific session of Kuban' Medical Institute), Krasnodar, 1957, p.98.

7. Rusakov A.V. Sud.-med. ekspert., 1930, no. 14, p. 3.

8. Serbilat'ev L.I. Russk. vrach, 1910, no. 46, p. 1752.

9. Shevlyagina M.I. Ter. arkh. 1939, vol. 17, no. 5, p. 87.

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ИЗМЕНЕНИЯ В СИСТЕМЕ СВЕРТЫВАНИЯ КРОВИ ПРИ ОТРАВЛЕНИИ УКСУСНОЙ КИСЛОТОЙ

L.N. Kl. n. Med. (Moskva) 47:84-7 (1969)

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При отравлении уксусной кислотой во внутренних органах нередко развиваются тромбозы и кровоизлияния (Л. И. Сербилатьев; А. В. Русаков; М. И. Шевлягина; Г. Г. Захаров; А. Ф. Рубежанский; А. С. Лонщакова и А. И. Зикеева; В. К. Дербоглав). Возникновение кровоизлияний связывают с повышенной сосудистой проницаемостью вследствие токсического поражения сосудистой стенки уксусной кислотой или рассматривают как результат поражения сосудов продуктами усиленного белкового распада, образующимися при ожоге тканей желудочно-кишечного тракта (М. И. Шевлягина). Механизм же возникновения внутрисосудистых тромбозов внутренних органов еще менее изучен.

В настоящее время ни один случай тромбоза сосудов не может быть рассмотрен без учета состояния свертывающей системы крови. Однако работ, освещающих состояние процессов гемокоагуляции при отравлении уксусной кислотой, мы в литературе не нашли. Анализ изменений, происходящих в системе свертывания крови при отравлении уксусной кислотой, позволит лучше разобраться в некоторых сторонах патогенеза болезни, определить причину ряда осложнений и, возможно, выработать более рациональную терапию.

Мы изучали изменения свертывающей системы крови у 70 больных с отравлением уксусной кислотой в возрасте от 14 до 76 лет. Мужчин было 19, женщин — 51. Однократное исследование гемокоагуляции проведено у 47 больных, в динамике — у 23. Для контроля взяты показатели коагулограммы 50 практически здоровых лиц (И. А. Коптева).

Определяли показатели общей коагулирующей активности крови (время свертывания по Ли и Уайту, время рекальцификации плазмы по Бергергофу и Рока, толерантность плазмы к гепарину по Поллеру), показатели I фазы гемокоагуляции (тромбопластиновая активность по Грехему, Лангделле и Бринкхоусу в модификации В. П. Балуда, антитромбопластиновая активность по В. П. Балуда и В. В. Черной), показатели II фазы (протромбиновый показатель по Туголукову, концентрация протромбина двухступенчатым методом по Вейр и Сигерсу в модификации В. П. Балуда, концентрация Ас-глобулина по Леви и Вейр, концентрация проконвертина по Оврену и Аас, антитромбиновая активность по Дель-Буано и Пазеро), показатели III фазы (концентрация фибриногена суховоздушным методом, активность фибринностабилизирующего фактора по В. П. Балуда, Н. А. Жуковой, Ж. И. Руказенковой, фибриноген В) и фибринолитическую активность по Ковальскому, Копек и Пиверовскому.

Наши исследования показали, что при отравлении уксусной кислотой происходят существенные сдвиги в функциональном состоянии свертывающей системы крови, характер которых в значительной мере определяется сроками, прошедшими с момента отравления. В связи с этим все больные были разделены на 2 группы: в 1-ю вошло 30 больных, исследование гемокоагуляции которых проводилось в основном в 1-е сутки после отравления; во 2-ю группу — 40 больных, обследованных на 2—3-и сутки.

У всех больных 1-й группы отмечено повышение общей коагулирующей активности крови, о чем свидетельствовало прежде всего статистически достоверное повышение толерантности плазмы к гепарину в среднем до 3,2 мин. ($P<0,001$; норма 4,3 мин.). Сокращение времени свертывания крови в среднем до 6,5 мин. ($P<0,05$; норма 7,1 мин.) и уменьшение времени рекальцификации в среднем до 94 сек. ($P<0,001$; норма 100 сек.) также указывают на повышенную свертываемость крови в этой группе больных. Клинически эта гиперкоагулемия проявляется в том, что при венепункции кровь нередко свертывается в шприце или даже в игле. Мы отмечали это и в тех случаях, когда взятие крови производилось силиконированными иглами и шприцами.

Исследуя остальные показатели коагулограммы, мы пришли к выводу, что повышение общей коагулирующей активности крови обусловлено прежде всего сдвигами в I фазе гемостаза. Из 26 обследованных больных этой группы только у 5 тромбопластиновая активность была в пределах нормы, а у остальных она оказалась повышенной в среднем до 65,7% при норме 47,7% ($P<0,001$). Следует отметить, что усиление тромбопластиновой активности нередко сочеталось с понижением антитромбопластиновой, которое наблюдалось у 17 из 26 обследованных.

Антитромбиновая активность крови у половины исследованных больных была снижена. Концентрация прокоагулянтов во II фазе свертывания крови (протромбиновый показатель, Ас-глобулин, проконвертин и протромбин) была близка к норме при легких отравлениях и несколько снижалась при более тяжелых.

Угнетение антикоагулянтной активности крови, безусловно, является одним из факторов, вызывающих наступление гиперкоагуляции крови.

Небезынтересно отметить, что повышение свертываемости крови у больных сочеталось с угнетением фибринолиза. Из 30 больных этой группы значительная его депрессия отмечена у 28 (от 5 до 15 часов при норме 3—5 часов). При гиперкоагулемии угнетение фибринолиза является неблагоприятным моментом. К тому же активность фибринностабилизирующего фактора у подавляющего большинства больных была повышена в $1^1/_2$—2 раза.

Выявлено повышение концентрации фибриногена в среднем до 485 *мг%* ($P<0,001$; норма 402 *мг%*).

Характерным для больных 2-й группы было понижение свертываемости крови, на что указывало статистически достоверное понижение толерантности плазмы к гепарину в среднем до 6 мин. ($P<0,001$), удлинение времени

рекальцификации плазмы до 132 сек. ($P<0,001$) и времени свертывания крови до 9 мин. ($P<0,001$).

Понижение общей коагулирующей активности крови сопровождалось у $^2/_3$ обследованных больных снижением активности тромбопластина. У отдельных больных последняя равнялась нулю, а у остальных она была близка к норме или повышена. Но, несмотря на повышение этого показателя, свертываемость крови как конечный результат процесса гемокоагуляции была снижена, так как почти у всех больных 2-й группы отмечено значительное повышение антитромбопластиновой и антитромбиновой активности крови: первая составляла в среднем 177% ($P<0,001$), вторая — 147% ($P<0,01$).

Концентрация прокоагулянтов во II фазе свертывания крови уменьшалась при тяжелых отравлениях.

Концентрация фибриногена была выше, чем у больных 1-й группы, равняясь в среднем 566 *мг%* ($P<0,001$). Фибринолиз у большинства обследованных (32 из 37) оставался значительно угнетенным. Активность фибринстабилизирующего фактора сохранялась на прежнем уровне, т. е. была повышена.

Система свертывания крови при отравлении уксусной кислотой претерпевает фазовые изменения. В первое время после отравления наблюдалось повышение свертываемости крови. Состояние ускоренной коагуляции было обусловлено прежде всего усилением тромбопластиновой и угнетением антикоагулянтной активности. При этом гиперкоагуляция в сочетании с угнетением фибринолиза, гиперфибриногенемией и усилением активности фибринстабилизирующего фактора создают предпосылки для развития внутрисосудистого тромбоза. Дополнительным подтверждением этого служит положительная реакция на фибриноген Б, отмеченная у 19 больных.

Фаза гипокоагуляции, сменяющая период повышенного свертывания крови, по-видимому, является ответной реакцией организма на угрозу внутрисосудистого тромбообразования.

Замедление процессов свертывания крови сохраняется у большей части больных и в более отдаленные сроки (1—2 недели), что, вероятно, способствует возникновению поздних желудочных кровотечений.

В заключение считаем необходимым отметить, что выраженность нарушений в системе свертывания крови в значительной мере коррелирует с тяжестью поражения.

ЛИТЕРАТУРА

Балуда В. П., Маляровский В. Н., Ойвин И. А. Лабораторные методы исследования свертывающей системы крови. М., 1962. — Дорболав Б. К. В кн.: Судебно-медицинская экспертиза и криминалистика на службе следствия. Грозный, 1962, в. 3, с. 285. — Захаров Г. Г. В кн.: Сборник научных работ Минск. мед. института. Минск, 1957, т. 18, с. 199. — Коптева И. А. В кн.: Схема лечения больного в терапевтической клинике. Куйбышев, 1957, с. 19. — Лопыдакова А. С., Зикеева А. И. В кн.: Проблема замедления крови применительно к хирургии. Алма-Ата, 1958, с. 158. — Рубежанский А. Ф. Тезисы докл. Научной сессии Кубанск. мед. ин-та. Краснодар, 1957, с. 98. — Русаков А. В. Суд.-мед. эксперт., 1960, № 14, с. 3. — Сербиладьев Л. И. Русск. врач, 1910, № 49, с. 1752. — Шелягина М. И. Тер. арх., 1939, т. 17, в. 5, с. 87.

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BLOOD COAGULATION SHIFTS IN ACETIC ACID POISONING

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Summary

The author studied the blood coagulation in 70 patients with acetic acid poisonig. Subject to determination were indices characterizing the general blood coagulation activity, first, second and third phases of blood coagulation, and fibrinolytic activity of the blood. During the first 24 hours after poisoring the blood coagulation increases. This is the result of increased thromboplastin and decreased anticoagulation activity. At the same period there were noted an augmented quantity of fibrinogen, a rise of the activity of the fibrin-stabilizing factor and inhibition of fibrinolysis. Subsequently (on the second — third day) a state of hypocoagulation develops. There occurs a significant rise of the activity of antithromboplastin and antithrombin, a decrease of the thromboplastin activity. The concentration of fibrinogen and fibrinase activity remain augmented, whereas fibrinolysis — inhibited.

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07:616.155.1-007.1-073.916

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Migration of Acetic Acid and Sodium Acetate and Their Effects on the Gastric Transmucosal Ion Exchange

By

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Abstract

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The kinetics of the disappearance of acetic acid and sodium acetate from the stomach during secretion and secretory arrest was studied in anesthetized cats. Model experiments were also performed in which the solutes diffused through a cellophane membrane. On instillation of acetic acid into non-secreting stomachs, the hydrogen ion concentration decreased significantly more rapidly than the acetate concentration ($p<0.05$). The increase in the combined sodium and potassium ion concentrations was significantly greater than the increase in the chloride ion concentration. Thus, in addition to molecular diffusion of acetic acid, exchange diffusion took place between hydrogen ions and sodium ions. A small amount of secretion masked this exchange. The acetate disappeared more rapidly on instillation of acetic acid than on instillation of sodium acetate. Acetic acid increases the ionic permeability of the gastric mucosa and has inhibitory effect on secretion. It has been reported that this secretory inhibition persists for up to 24 hrs. By repeated instillation experiments in the cat it was found that the permeability effects of a 30 min installation of 170 mM acetic acid did not persist for longer than 90 min. Sodium acetate per se probably had no effect on the ionic permeability of the gastric mucosa. It is suggested that the permeability increasing effect of acetic acid is related to transient intracellular accumulation of the diffusing acid. Epithelial abrasion that would give rise to an increased permeability by decreasing the diffusion distance requires restoration of the surface epithelium within 90 min and in fact, no abrasion was observed as an affect on acetic acid on the mucosa.

The permeability of the gastric mucosa to different substances has attracted great attention because of its important pharmacological aspects. Teorell (1939) found that on instillation into non-secreting cat stomachs weak acids disappeared more rapidly than strong acids. He suggested that weak acids in an undissociated and thus in a more lipid soluble form could permeate more rapidly through the "non-aqueous parts of the cell membrane". Schanker *et al.* (1957) showed that weak electrolytes rapidly diffused out of the rat stomach if the stomach pH was such that the electrolytes were undissociated. It has been suggested by Martin (1963) that a weak acid which diffuses in an undissociated form from the acid environment in

the stomach lumen accumulates intracellularly in the mucosal cells, where the pH is higher.

It has been found that several weak acids increase the permeability of the gastric mucosa to ions. Davenport (1964) found that acetic acid increased the net flow of water, hydrogen ions, sodium ions, potassium ions and chloride ions through the mucosa. He obtained a similar effect with propinic, butyric and acetylsalicylic acids. Solutions of the same acids buffered with TRIS and glycine to pH of about 7 had no effect on the flows. Flemström, Frenning and Öbrink (1964) showed that after instillation of 170 mM acetic acid, the permeability coefficient for hydrogen ions on subsequent installation of hydrochloric acid was increased by 100 %.

Babkin, Hebb and Kreuger (1941) studied the effect of acetic acid instillation in dogs with an oesophagostomy and gastric fistula. The stomach was stimulated to secretion by sham-feeding or subcutaneous injection of histamine. After instillation of 1 % (i.e. 167 mM) acetic acid, there was a reduced secretion volume, lower acidity and a reduced "peptic power". The acidity value regained the normal level after 24 hrs, while the volume remained lower for several days. Grant (1944, 1945) demonstrated epithelial abrasion after instillation of acetic acid, but she also found that several ordinary food substances had a similar effect.

In view of these results it seemed of interest to study in detail the kinetics of the disappearance of acetic acid and sodium acetate after their instillation into the stomach, and to investigate the duration of their effect on the permeability of the mucosa to ions.

Methods

Experimental animals

The experiments were performed on cats weighing between 1.5 and 3.8 kg. The cats were starved for at least 18 hrs before the experiments in order to produce a non-secretory state. They were anesthetized initially with chloroform and anesthesia was then maintained by a mixture of chloralose and urethan (1:10) given i.v. A midline incision was made and the stomach isolated by means of ligature at the cardia and pylorus, care being taken not to disturb the blood supply to the stomach. A glass tube was then inserted into the pyloric end of the stomach, after which the abdomen was closed. The secretory state of the stomach was subsequently checked over a period of about 2 hrs or more. The stomach was considered to be in a state of secretory arrest when during a period of 1 hr before the start of the experiment the amount of secretion did not exceed 0.4 ml and the acidity 10 mM. Some experiments were also performed on stomachs which were secreting "spontaneously".

Analyses

Acidity determination. 0.05 ml samples were pipetted into 4 ml double distilled water and titrated with 0.01 M NaOH (indicator bromthymolblue). The coefficient of variation was ±1 % at 100 mM.

Chloride was determined electrometically with silver-silver chloride electrodes on the same samples as were used for the acidity determination. 0.005 M $AgNO_3$ was used for titration. The coefficient of variation was ±0.5 % at 100 mM.

Sodium and potassium were determined flamephotometrically (flame photometer Eppendorf, Netheler and Hinz GMBH, Hamburg) after dilution of 0.05 ml samples in double distilled water. A propane-air flame was used. The coefficient of variation was ±2 % at 100 mM (for sodium).

Acetate determination. ^{14}C-labelled solutions of acetate and acetic acid were used. For analysis ^{14}C was converted into $^{14}CO_2$ by a combustion method described by Kalberer and

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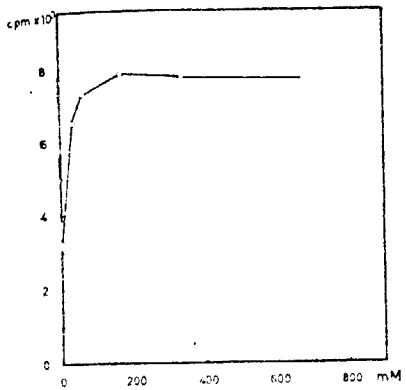

Fig. 1

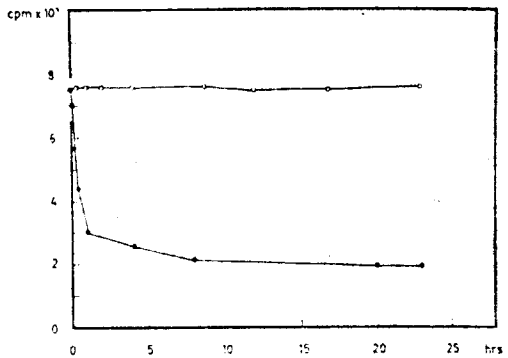

Fig. 2

Fig. 1. The activity in samples from the same ^{14}C-labelled acetic acid solution, which had been burnt according to Kalberer and Rutschmann on paper pretreated with NaOH of different strengths. The figure shows the activity as a function of the strength of the NaOH solution used for pretreatment of the paper.

Fig. 2. The activity in samples from the same ^{14}C-labelled acetic acid solution, which had been burnt after different drying times. The upper curve shows the result when paper prepared with 340 mM NaOH was used. The lower curve shows the result with unprepared paper.

Rutschmann (1961) and counted by liquid scintillation (Packard TriCarb 314 EX). In order to avoid evaporation of the volatile acetic acid, combustion paper (Filter paper No. 589, Schleicher and Schuell AG, Feldmeilen: Zürich, Switzerland), which had been previously soaked in NaOH solution and allowed to dry, was used. The CO_2 was absorbed in 15 ml of a solution made by mixing 120 ml distilled 2-aminoethanol and 880 ml methanol. 10 ml of this solution was then mixed with 10 ml scintillation liquid (toluene containing 4.0 g/L PPO (2,5-diphenyloxazol) and 0.1 g/L POPOP (1,4-di 2 (5-phenyloxazolyl) benzene)). The optimal NaOH concentration for pretreatment of the combustion papers was determined by burning samples from a 170 mM, ^{14}C-labelled acetic acid solution on paper which had been pretreated with NaOH of different strengths. The results are given in Fig. 1. The counting rate showed a plateau above approximately 0.17 m NaOH and 0.34 M NaOH was selected. A comparison was also made of the effect of the length of drying time on the radioactivity of samples when prepared and unprepared paper was used. These results are shown in Fig. 2, which gives the number of cpm as a function of the drying time. With prepared paper the counting rate was constant for drying times varying between 20 min and 24 hrs. With unprepared paper a considerable loss of activity was obtained. The samples were burned on prepared paper after drying in room temperature for 10 - 20 hrs. The quantity loaded on the paper was 0.05 ml and the coefficient of variation for the counting rate was ±1.5 %; the expected statistical error for the same number of impulses due to the randomly distributed disintegrations being 0.3 %. The stability of the counter was tested by counting a ^{14}C sample 60 times during a period of about 10 hrs. The coefficient of variation was ±0.63 % (expected statistical error ±0.3 %) *i.e.* the stability of the apparatus was good.

General experimental procedure

6 ml of the test solution was instilled into the stomach in all cat experiments. Every fifteenth min the fluid in the stomach was drained, its volume measured and samples taken for analysis. A total of 0.1 ml was used for determination of acidity, chloride, sodium and potassium. A further 0.05 ml was used for acetate determination. After this procedure, which took 3–4 min, the fluid remaining was reinstilled into the stomach.

The experiments were begun by the installation of 170 mM hydrochloric acid for two or four 15 min periods (*i.e.* the effective installation time for the same solution was 30 or 60 min respectively), except in some experiments in which acetic acid was instilled into stomachs during "spontaneous" secretion. A 30 or 60-min installation of 170 mM acetic acid or sodium

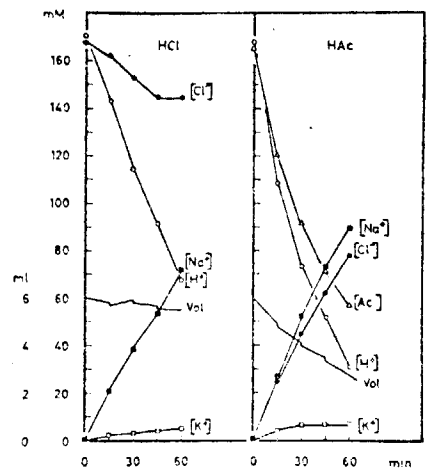

Fig. 3

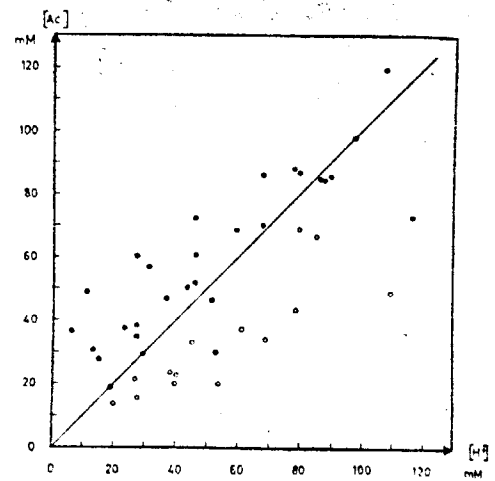

Fig. 4

Fig. 3. To the left, the result of installation of 170 mM hydrochloric acid into a non-secreting cat stomach. To the right, the result of installation of 170 mM acetic acid into the same stomach.

Fig. 4. The acetate concentration plotted against the hydrogen ion concentration in the same sample. The circles represent values from installations in "spontaneously" secreting stomachs and the dots values from instillations into non-secreting stomach. The identity line Ac = H is drawn in.

acetate solution was then given. To study the duration of the effect of acetic acid on the gastric mucosa, three 30-min instillations of hydrochloric acid were given after the instillation of acetic acid. This latter type of experiment was also performed with sodium acetate. In control experiments 5 consecutive 30-min instillations of 170 mM hydrochloric acid were given.

Model experiments

12 ml of the test solution (170 mM HCl, HAc or NaAc) were placed in one chamber of a diffusion cell separated from 4000 ml 170 mM NaCl by a cellophane membrane. The surface of the membrane was 16.5 cm². Samples were taken from the test solution every 15th min for 1 hr. Both compartments were stirred continuously.

Results

Diffusion of acetic acid and sodium acetate

6 ml of 170 mM hydrochloric acid was first instilled into a non-secreting stomach. One hr later, the stomach contents were removed and 6 ml of 170 mM acetic acid or sodium acetate solution were instilled and analyses made over a similar time period. In some experiments acetic acid was instilled into stomachs which exhibited "spontaneous" secretion. The results are shown in Fig. 3, 4 and 5 and in Table I and II.

The reduction in hydrogen ion concentration was greater during instillation of acetic acid than during instillation of hydrochloric acid into non-secreting stomachs (see Fig. 3). There was a considerable decrease in volume of the gastric contents

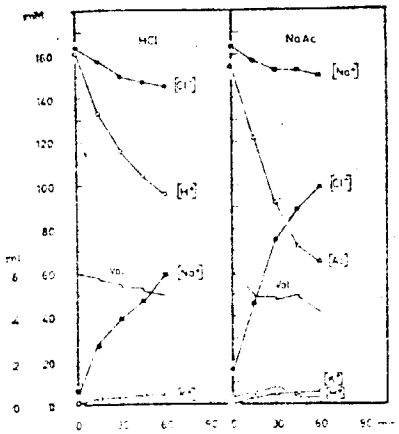

Fig. 5

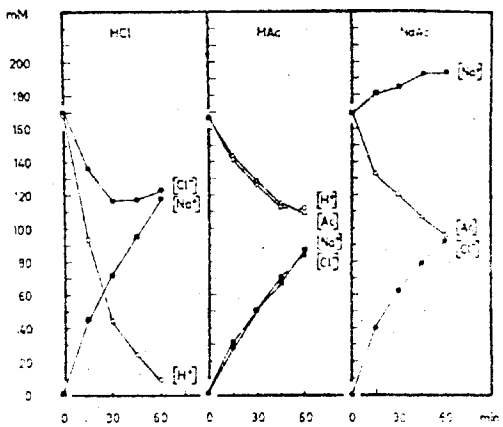

Fig. 6

Fig. 5. Instillation of 170 mM hydrochloric acid and of sodium acetate solution in the same non-secreting cat stomach.

Fig. 6. Model experiment in which 12 ml 170 mM test solution was dialysed against 4000 ml 170 mM sodium chloride solution through a cellophane membrane.

during instillation of acetic acid: this was probably due to the hypotonicity of the solution. The hydrogen ion concentration declined more rapidly than that of acetate. The combined sodium and potassium influx into the stomach was more rapid than that of chloride. The difference between the reduction in hydrogen ion concentration and the reduction in acetate concentration after 60 min was, on the average, 14 mM. The corresponding difference between the combined increase in sodium ion and potassium ion concentration and the increase in chloride ion concentration was 9 mM. These differences were statistically significant ($p<0.05$ in both cases).

On instillation of acetic acid into "spontaneously" secreting stomachs the acetate concentration decreased more rapidly than that of the hydrogen ions. The chloride ion concentration increased more quickly than the combined sodium ion and potassium ion concentrations. In other words the situation was the reverse of that observed on instillation into non-secreting stomachs. Thus even a very low secretion low secretion rate will tend to diminish the differences between ΔH and ΔAc and between $\Delta(Na+K)$ and ΔCl observed in non-secreting stomachs (see Table I). (For a general discussion on the influence of hydrochloric acid secretion on the apparent hydrogen ion permeability, see Öbrink and Waller 1965).

The results obtained from instillations of acetic acid into secreting and non-secreting stomachs are presented in Fig. 4.

Fig. 5 shows the results of a typical experiment with instillation of 170 mM sodium acetate solution into a non-secreting stomach. The sodium concentration decreased very slowly, while the increase in potassium concentration was of the same order size as with acetic acid. The hydrogen ion concentration increased on the average

Table I. Changes in composition of the test solution on instillation of 6 ml 170 meq/l acetic acid into the cat stomach during 1 hr.

Secretory condition	ΔH mM	ΔAc mM	$\Delta(H-Ac)$ mM	ΔNa mM	ΔK mM	ΔCl mM	$\Delta(Na+K-Cl)$ mM	Δv ml
	−150	−133	−17	+104	+6	+99	+11	−2.1
	−153	−141	−12	+99	+5	+92	+12	−0.5
	−160	−130	−30	+112	+5	+98	+19	−2.6
Arrest	−145	−137	−8	+95	+4	+91	+8	−1.5
	−152	−153	+1	+95	+6	+104	−3	−1.3
	−137	−111	−26	+84	+7	+78	+11	−3.1
	−158	−155	−3	+93	+6	+98	+1	−0.7
Mean	−151	−137	−14	+97	+6	+94	+9	−1.7
	−124	−144	+20	+76	+7	+101	−17	+3.5
Spontaneous	−122	−142	+20	+74	+9	+110	−27	+3.8
secretion	−142	−155	+13	+101	+8	+126	−11	+1.4
	−146	−153	+7	+109	+6	+115	−2	+1.2
Mean	−134	−149	−15	+90	+8	+113	−15	+2.5

by 3 mM ($n=3$). The acetate concentration decreased relatively rapidly, but more slowly than when acetic acid was instilled. The chloride ion concentration increased rapidly. There was a mean volume decrease in the gastric contents of 1.2 ml.

Model experiments

The results are presented in Fig. 6 and Table II.

Model experiments with 170 mM hydrochloric acid gave, essentially, the same result as instillations into cat stomachs. In experiments with 170 mM acetic acid the decrease in hydrogen ion concentrations was smaller than in experiments with hydrochloric acid. The reduction in hydrogen ion concentration was equal to the reduction in acetate concentration. The increase in sodium ion concentration equalled the increase in chloride ion concentration.

Table II. Model experiments in which 12 ml 170 mM inner solution was dialysed against 4000 ml 170 mM NaCl across a cellophane membrane, for one hr. The table gives the changes in concentration in the inner solution (mM) ± S.E.

Inner solution	ΔH mM	ΔCl mM	ΔNa mM	ΔAc mM	n
HCl	−160 ± 2	−45 ± 2	+113 ± 2	—	7
HAc	−81 ± 4	+92 ± 3	+95 ± 4	−78 ± 4	5
NaAc	—	+96 ± 2	+21 ± 2	−76 ± 3	3

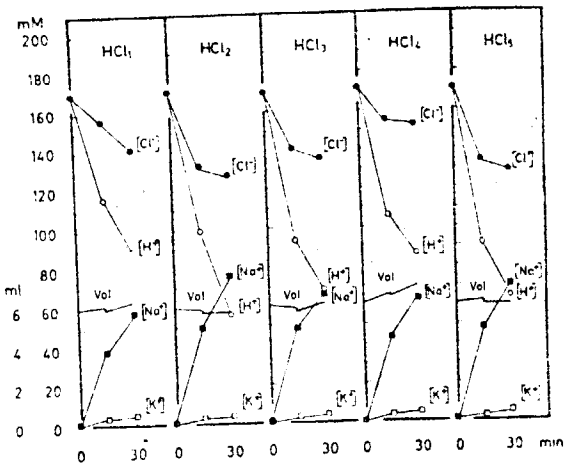

Fig. 7. Result of a typical experiment with one hydrochloric acid installation (HCl_1) *before* and three consecutive hydrochloric acid installations (HCl_2—HCl_5) *after* a 30 min instillation of 170 mM acetic acid. In this experiment one further acetic acid instillation followed by a hydrochloric acid instillation (HCl_5) was given.

In experiments with sodium acetate the decrease in acetate concentration was of the same order of size as in experiments with acetic acid. The sodium concentration increased, probably because the chloride ion concentration increased more rapidly than the acetate concentration decreased, *i.e.* there was a diffusion effect (*cf.* Teorell 1935 and 1937.

The effects of acetic acid and sodium acetate on the ionic permeability of the gastric mucosa and their duration

170 mM hydrochloric acid was first instilled into a non-secreting cat stomach. Thirty min later the gastric contents were removed and were replaced by a similar volume of 170 mM acetic acid or sodium acetate solution. Finally, repeated hydrochloric acid instillations were given. When changing the instillation solution the stomach was emptied as completely as possible. The results are shown in Fig. 7 and 8, and Table III and IV.

After a previous instillation of acetic acid the reduction in hydrogen ion concentration on instillation of hydrochloric acid was greater than if not preceded by acetic acid. The sodium concentration increased more than before the acetic acid instillation, and the decrease in chloride ion concentration was greater. The increase in potassium concentration was unchanged.

With subsequent hydrochloric acid instillations the changes in concentration of all ions except the potassium decreased. The average concentration changes approached those observed during the initial hydrochloric acid instillation. The volume changes were of the same order of magnitude throughout. In a few experiments the instillation solutions darkened somewhat as an evidence of minor bleeding. This did however, only happen during the last two hydrochloric acid instillations.

In the experiment illustrated in Fig. 7, a further acetic acid instillation was given

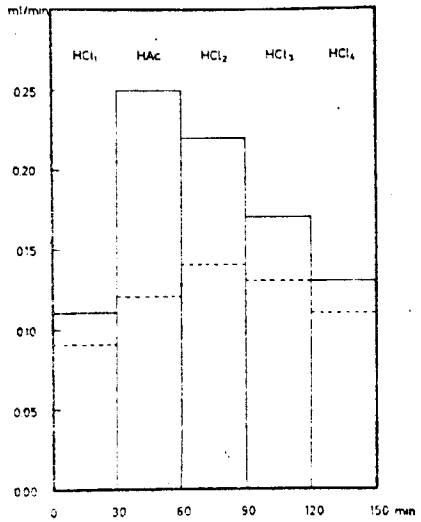

Fig. 8

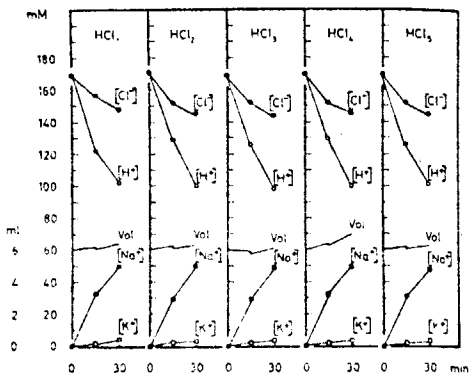

Fig. 9

Fig. 8. The mean permeability coefficients for hydrogen ions and sodium ions from eight experiments of the same type as in Fig. 7. The coefficients for sodium ions are shown by the broken lines.

Fig. 9. Result of an experiment with repeated instillations of 170 mM hydrochloric acid in the same non-secreting cat stomach.

TABLE III. Changes in concentration and volume on 30 min instillations of 170 mM hydrochloric acid into the cat stomach at secretory arrest. A 30 min instillation of 170 mM acetic acid was given between the first and second instillation of hydrochloric acid. The table gives the mean values of 5 expts., ± S.E.

Installation no.	ΔH mM	ΔCl mM	ΔNa mM	ΔK mM	ΔV ml
1.	− 71 ± 4	− 24 ± 2	+ 46 ± 4	− 5 ± 1	+ 0.4 ± 0.1
2.	− 114 ± 6	− 44 ± 1	+ 70 ± 4	+ 5 ± 1	+ 0.4 ± 0.4
3.	− 98 ± 2	− 35 ± 1	+ 65 ± 3	+ 4 ± 1	+ 0.5 ± 0.2
4.	− 81 ± 4	− 29 ± 1	+ 56 ± 5	+ 4 ± 1	+ 0.4 ± 0.3

when the permeability had returned to the normal level. A new increase in permeability was observed.

Sodium acetate had a considerably smaller effect on the ionic permeability of the gastric mucosa.

When hydrochloric acid is instilled into a non-secreting stomach, the hydrogen ion concentration decreases according to the equation

$$H = H_0 e^{\frac{-kt}{p}}$$

TABLE IV. The permeability coefficients (ml/min) for hydrogen ions (k) and sodium ions (r) on instillation of 170 mM hydrochloric acid before and after instillation of 170 mM acetic acid and of sodium acetate solution.

HCl 1		Interspaced	HCl 2		HCl 3		HCl 4	
k	r	instillation	k	3	k	r	k	r
0.13	0.10		0.22	0.16	0.19	0.15	0.11	0.12
—	—		0.23	0.13	0.17	0.11	0.15	0.12
0.09	0.06		0.24	0.15	0.18	0.13	0.16	0.10
0.09	0.06	Acetic acid	0.21	0.11	0.16	0.11	0.10	0.06
0.13	0.10		0.22	0.14	0.19	0.13	0.15	0.13
0.12	0.08		0.18	0.13	0.16	0.12	0.10	0.10
0.08	0.08		0.20	0.13	0.15	0.11	0.13	0.11
0.11	0.15		0.22	0.16	0.16	0.14	0.11	0.14
0.11	0.09	Mean	0.22	0.14	0.17	0.13	0.13	0.11
0.11	0.09	Sodium	0.13	0.09	0.13	0.09	0.13	0.09
0.10	0.08	acetate	0.13	0.09	0.10	0.07	0.10	0.07
0.08	0.10		0.12	0.11	0.09	0.10	0.10	0.09
0.10	0.09	Mean	0.13	0.10	0.11	0.09	0.11	0.08

where H is the observed ion concentration (mM) at time t, H_0 the original hydrogen ion concentration, k the permeability coefficient for hydrogen ions (ml/min), and p the volume (ml).

The sodium concentration increases according to the equation

$$Na = S_0 \left(1 - e^{\frac{-rt}{p}}\right)$$

where Na is the actual sodium concentration at time t, S_0 the equilibrium concentration and r the permeability coefficient for sodium ions. The value for S_0 is given as 140–150 mM (Teorell 1947, Öhrink 1948).

In the present calculations the value 145 mM was used. The values for k and r from instillations of hydrochloric acid before and after instillation of acetic acid or sodium acetate are given in Table IV and Fig. 8. An instillation of acetic acid increased the permeability coefficient for hydrogen ions by an average of 100 %. The corresponding increase in the permeability coefficient for sodium ions was 55 %. No significant difference was found between the permeability coefficients obtained on instillation of hydrochloric acid 60–90 min after the instillation of acetic acid and those obtained at the initial hydrochloric acid instillation. The effect of the acetic acid on the ionic permeability of the gastric mucosa was thus reversible and did not persist for longer than about 90 min.

Control experiments

In order to ascertain the extent to which residual solution from the immediately preceding instillation influenced the result, samples were taken 2 min after instillation of a new solution. The changes in concentration attributable to 2 min diffusion were calculated and subtrated from those obtained in the analyses. It was found that

the hydrogen ion concentration decreased and the sodium ion concentration increased by only a few mM as a result of dilution. The effect of dilution was thus negligible.

In order to exclude the possibility that the permeability altered spontaneously during the experimental period, tests were carried out with five consecutive 30 min instillations of 170 mM hydrochloric acid. The result of such a test is shown in Fig. 9. The changes in concentration and the permeability coefficients were the same in all 5 instillations (range for the permeability coefficient ± 0.01 ml/min).

Microscopic examination

Three cats were anesthetized and operated on in the usual way. At the operation a specimen from the corpus of the stomach was taken for histological examination, after which 170 mM acetic acid was instilled into the stomach. 30 min later a further specimen was taken from the stomach wall. The specimens were immediately immersed in 10 % formalin. Hematoxylin and eosin stains were used. No difference was observed between sections from the two specimens.

Discussion

Migration of acetic acid and sodium acetate

Concerning the changes in ion concentration, the results obtained in model experiments with 170 mM hydrochloric acid were essentially the same as those obtained in the animal experiments, indicating that the mechanism of migration of hydrochloric acid was the same in both cases. This was first shown by Teorell (1947).

On instillation of acetic acid into non-secreting stomachs and in model experiments, there was an outward diffusion of acetic acid and an inward diffusion of sodium, potassium and chloride ions. In the model experiments the disappearance rate of acetic acid was lower than that of hydrochloric acid. This was contrary to what was found in the animal experiments in which the disappearance rate of acetic acid was the highest. These results indicate different mechanism of migration of the acid through the gastric mucosa and the cellophane membrane. It is in accordance with the theory that lipid soluble undissociated acids can pass through the lipid regions of the cell membrane. In the animal experiments the decrease in hydrogen ion concentration was significantly greater than the decrease in acetate concentration. The combined sodium and potassium ion concentration increased significantly more rapidly than the chloride ion concentration. This suggests that apart from molecular disappearance of acetic acid there was an exchange between hydrogen ions and sodium-potassium ions. The presence of secretion tended to mask the magnitude of this ion exchange. Evidence for this is given in Fig. 4 and Table I. After instillation of acetic acid into cat stomach, the volume of the intragastric solution decreased, which was probably due to the hypoosmolarity of the acid.

On instillation of sodium acetate into the stomach and in model experiments, an acetate-chloride exchange occurred. In the model experiments, where the sodium concentrations in both compartments were initially the same, the sodium concen-

tration increased in the inner solution (Fig. 6 and Table II). This was probably a diffusion effect, since the mobility of the chloride ions is greater than that of the acetate ion (7.9×10^{-4} and 4.3×10^{-4} cm/sec, respectively, in infinite dilution at 25° C). In instillations into the cat stomach the situation was more complicated due to the existence of a concentration difference between "inner and outer solution" (170 mM - S^o, i.e. about 145 mM). Experimentally the sodium concentration decreased somewhat (Fig. 5), which can be interpreted as the resultant of simultaneous differences in ionic mobilities and in concentration.

Effects of acetic acid and sodium acetate on the gastric transmucosal ion exchange

An important factor in the regulation of the acidity in the stomachs is the exchange diffusion between sodium and hydrogen ions (Teorell 1947, Öbrink 1948). On instillation of hydrochloric acid into a cat stomach the difference between the decrease in hydrogen ion concentration and the decrease in chloride ion concentration was approximatively equal to the increase in sodium and potassium concentration both before and after instillation of acetic acid. The net result of the effect of acetic acid on the mucosa may be interpreted thus as an increased diffusion exchange between hydrogen and sodium ions.

On the basis of the theory that lipophilic undissociated acids pass through the lipid regions of the cell membranes, it has been suggested that a varying degree of dissociation takes place intracellularly (dependent on the pK_a value of the acid). This would result in an intracellular liberation of hydrogen ions and, assuming that the efflux/metabolism is low, an anionic accumulation. Accumulation of ions in the cells would give rise to a transient intracellular pH-reduction, intracellular hypertonicity and swelling of the mucosa. We suggest that these changes might be the causes of the permeability increasing effect of acetic acid. It agrees well with the fact that the mucosal permeability for ions returned to normal in a relatively short time. Pathways for migration of sodium ions into the gastric lumen are not known but evidence has been presented that the disappearance of hydrogen ions takes place in an extracellular route (Öbrink and Waller 1965). It is possible that swelling of the mucosa increases the extracellular space. An increased blood flow in the mucosa due to transient local acidosis or histamine liberation (cf. Johnson 1966, Johnson and Overholt 1967) may also be of importance of the rate of the exchange diffusion. The reversibility of the effect of acetic acid on the ionic permeability of the gastric mucosa would seem to mean that no further hydrogen ions enter the cells when the acetic acid instillation is discontinued.

It has been reported by Grant (1945) that acetic acid produces local erosions of the surface epithelium of the mucosa. Such erosions would diminish the diffusion distance and thereby increase the permeability. It seems improbable, however, that such epithelial damage could be reversed during the 90 min period in which the stomach was in contact with 60—170 mM hydrochloric acid after exposure to acetic acid. No epithelial changes were observed on mucosa, which for 30 min had been in contact with acetic acid.

It has been suggested that acetic acid stimulates the stomach to secretion of neutralizing mucus (Babkin 1950). Evidence has been presented that acetic acid instillation gives rise to an increased flow of serum proteins and blood into the gastric lumen (Davenport 1966). As no volume increase as an effect of acetic acid instillation was obtained (see Table III), it is considered less probable that an inflow of neutralizing fluids could account for the increased disappearance rate of hydrogen ions.

Compared with acetic acid, sodium acetate had a small permeability increasing effect on the gastric mucosa. This could have been due to that the stomachs were not in a state of absolute secretory arrest (see Fig. 4), which would have meant the formation of a small amount of acetic acid from sodium acetate and hydrochloric acid. Sodium acetate per se probably had no effect on the mucosal permeability.

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References

BABKIN, B. P., *Secretory mechanism of the digestive glands*. 2nd ed. New York 1950.

BABKIN, B. P., C. O. HEBB and L. KREUGER, Changes in the secretory activity of the gastric glands resulting from the application of acetic acid solutions to the gastric mucosa. *Quart. J. exp. Physiol.* 1941. *31*. 63—78.

DAVENPORT, H. W., Gastric mucosal injury by fatty and acetylsalicylic acids. *Gastroenterology* 1964. *46*. 245—253.

DAVENPORT, H. W., Fluid produced by the gastric mucosa during damage by acetic and salicylic acid. *Gastroenterology* 1966. *50*. 487—499.

FLEMSTRÖM, G., B. FRENNING and K. J. ÖBRINK, The disappearance of acetic acid and acetate from the cat's stomach and its influence on the permeability for hydrochloric acid. *Acta physiol. scand.* 1964. *62*. 422—428.

GRANT, R., Conditions under which the epithelial cells of the gastric mucosa are shed and disintegrate. *Canad. med. Ass. J.* 1944. *51*. 577—578.

GRANT, R., Rate of replacement of the surface epithelial cells of the gastric mucosa. *Anat. Rec.* 1945. *91*. 175—185.

JOHNSON, L. R., Histamin liberation by gastric mucosa of pylorus-ligated rats damaged by acetic or salicylic acids. *Proc. Soc. exp. Biol.* (N.Y.) 1966. *121*. 384—386.

JOHNSON, L. R. and B. F. OVERHOLT, Release of histamine into gastric venous blood following injury by acetic or salisylic acids. *Gastroenterology* 1967. *52*. 505—509.

KALBERER, F. and J. RUTSCHMANN, Eine Schnellmetode zur Bestimmung von Tritium, Radiokohlenstoff und Radioschwefel in beliebigem organischen Probenmaterial mittels der Flüssigkeits-Scintillations-Zählers. *Helv. chim. Acta.* 1961. *44*. 1956—1966.

MARTIN, B. K., Accumulation of drug anions in gastric mucosal cells. *Nature* (Lond.) 1963. *198*. 896—897.

ÖBRINK, K. J., Studies on the kinetics of the parietal secretion of the stomach. *Acta physiol. scand.* 1948. *15*. Suppl. 51.

ÖBRINK, K. J. and M. WALLER, The transmucosal migration of water and hydrogen ions in the stomach. *Acta physiol. scand.* 1965. *63*. 175—185.

SCHANKER, L. S., P. A. SHORE, B. B. BRODIE and C. A. M. HOGBEN, Absorbtion of drugs from the stomach. I. The rat. *J. Pharmacol. exp. Ther.* 1957. *120*. 528—539.

TEORELL, T., Studies on the "diffusion effect" upon ionic distribution. I. Some theoretical considerations. *Proc. nat. Acad. Sci.* (Wash.) 1935. *21*. 152—161.

TEORELL, T., Studies on the "diffusion effect" upon ionic distribution. II. Experiments on ionic accumulation. *J. gen. Physiol.* 1937. *21*. 107—122.

TEORELL, T., On the stomach permeability for acids and some other substances. *J. gen. Physiol.* 1939. *23*. 263—274.

TEORELL, T., Electrolyte diffusion in relation to the acidity regulation of the gastric juice. *Gastroenterology* 1947. *9*. 425—445.

Acta physiol. scand. 1964. 62. 422—428

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The Disappearance of Acetic Acid and Acetate from the Cat's Stomach and its Influence on the Permeability for Hydrochloric Acid

By

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Abstract

Ehrström, G., B. Frenning and K. J. Öhrink. *The disappearance of acetic acid and acetate from the cat's stomach and its influence on the permeability for hydrochloric acid.* Acta physiol. scand. 1964. 62. 422—428. [illegible] The disappearance of acetic acid and acetate from the stomach and its effect on the gastric permeability for HCl [illegible] experiments with anesthetized cats [illegible] the hydrochloric acid secretion of the stomach. The [illegible] The acidity of the gastric juice [illegible] and reaches lower values. There is also a large [illegible] A mixture of sodium acetate and hydrochloric acid was found to have a similar effect on the permeability for hydrochloric acid.

Since the work of Teorell (1933) on the permeability of the gastric mucosa much evidence has appeared that the regulation of the gastric juice acidity is due to a great [illegible] to permeation of hydrogen, sodium, potassium and chloride ions. For a review of these papers the reader is referred to Heinz and Öhrink (1954). In 1939 Teorell also analyzed the permeability properties of a non-secreting stomach for several organic acids, as well as for some other substances. It should be noted that, in his work on weak acids, he found that permeability coefficients were higher for the weaker acids.

Interest in the penetration of weak acids and bases has occurred repeatedly in the literature. The studies on secretion of dyes and drugs are noteworthy (see for instance [illegible] 1957, Schanker *et al.* 1957).

[illegible] found that weak bases are accumulated in the gastric juice whereas [illegible] in the pancreatic juice the opposite seems to take place. This has been explained due to penetration of these weak bases or acids in undissociated

form. Diffusion is thought to take place not only between the cells, but also through the lipoid layers.

Babkin and his school paid special attention to the effect of acetic acid on the stomach (Babkin, Hebb and Kruse 1941, Babkin 1950). They showed that this acid could be a powerful stimulant for gastric mucus (Grant 1942). The rapid neutralization of acetic acid in the stomach could therefore be expected to be a result both of a high permeability coefficient and of the effect of the increased mucus secretion.

The effect of acetic acid may, however, be much more complicated. Gorbunova, Lebedinskaia and Savich (1933, cited from Babkin *et al.* 1941) introduced acetic acid into the main stomach of Pavlov pouch dogs and observed a decrease in the secretory response from the pouch after a test meal. Babkin *et al.* (1941) repeated these experiments and found that the diminution of the hydrochloric acid response occurred also after histamine stimulation. They could even obtain a persistent effect of the acetic acid treatment.

All the above experiments make it especially interesting to study in more detail the permeability properties of the gastric mucosa towards the acetic acid and acetate, as well as the possible influence of these substances on the permeability coefficient for hydrochloric acid.

The experiments to be reported are a preliminary work on these topics.

Methods

Experimental animals.

The experiments were performed on anaesthetized cats, (chloralose-urethane 1:10 i. v.) that had been fasted for at least 12 hours. After a midline incision, ligatures were tied around the pylorus and pylorus, care being taken not to interrupt the blood supply to the stomach. A glass cannula was introduced into the pyloric portion. Through this cannula a rubber tube could be used for filling or emptying the stomach. After completion of the surgical operation the animal was left for one, two or more hours, during which time it could be tested whether the anesthesia was at a correct level. A test solution was then introduced and remained in the stomach for a given length of time. After this period the volume was completely removed, measured and retitrated after a small sample had been taken for analysis.

The stomach was rinsed with the fluid to be introduced before every new experiment was started.

Analytical procedures.

Acidity determination. 0.1 ml sample was put into 4 ml of distilled water and titrated with 0.01 M NaOH, using bromthymol blue as an indicator. That means that only "total acidity" was determined as this indicator changes its colour between pH 6.0 and 7.5.

Chloride determination was made electrometrically with a silver chloride electrode on the same samples as used for the acidity determination. For titration 0.005 M $AgNO_3$ was used.

Osmolarity determination was made from freezing point depression measurement with the osmometer of "Advanced Instruments Inc.", Newton Highlands, Mass., U. S. A.

Three types of experiments were performed:

1. Introduction of 170 mM (352 mOsm) HCl followed by introduction of 170 mM (168 mOsm) HAc in the same cat.
2. Consecutive experiments on the same cat with a) 170 mM HCl b) 170 mM HAc and c) 170 mM HCl.
3. Consecutive experiments on the same cat with a) 170 mM HCl b) 170 mM HCl to which was added 170 m-moles per liter solution of sodium acetate in powder form (483 mOsm) c) 170 mM HCl.

In all types of experiments 6 ml of the test solution were introduced.

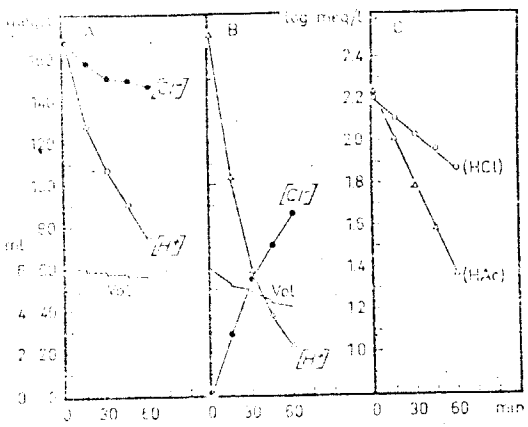

Fig. 1. A typical experiment with instillation of hydrochloric acid in a cat's stomach, followed by instillation of acetic acid. A: 6 ml of 170 mM hydrochloric acid. B: 6 ml of 170 mM acetic acid. C: The hydrogen ion concentrations plotted on a semilogarithmic scale.

TABLE I. Instillation of 6 ml hydrochloric acid and acetic acid respectively in a cat's stomach and an experiment with a model, where 12 ml of the test solution were allowed to diffuse against 60 ml of 170 mM sodium chloride through a cellophane membrane. k = the permeability coefficient for hydrogen ions (ml · min^{-1}); ΔV = ml is the change in volume per 60 min; HH (mM) = the decrease in total acidity in the first 60 min

Exp. no.	k_{HCl}	k_{HAc}	ΔV_{HCl}	ΔV_{HAc}	HH_{HCl}	HH_{HAc}
1	0.11	0.34	+ 0.5	− 1.3	117	153
2	0.06	0.13	0.3	− 1.6	85	166
3	0.09	0.35	+ 0.4	− 1.3	107	163
4	0.06	0.17	0	− 1.5	95	148
Mean value	0.08	0.26	+ 0.2	− 1.6	101	161
Model exp.	0.05	0.02			40	17

Results

A. Comparison of the permeability properties for hydrochloric acid and acetic acid in the same stomach.

Six ml of 170 mM HCl were introduced into the stomach and samples taken every 15 min. The resulting concentration of the hydrogen ion is seen in Fig. 1A. A consecutive introduction of 6 ml 170 mM HAc into the same stomach showed a similar disappearance, as can also be seen in Fig. 1B. The disappearance rate of acetic acid was always greater than that of hydrochloric acid. See Table I. This table also summarizes similar experiments performed in a model where hydrochloric acid and acetic acid were allowed to diffuse against 170 mM NaCl through a cellophane membrane. It

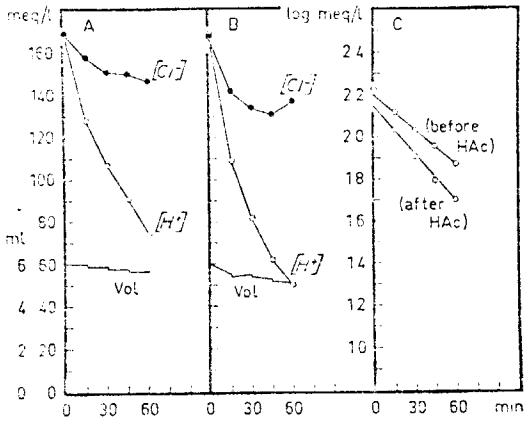

Fig. 2. An experiment with two instillations of hydrochloric acid on the same cat interspaced by an instillation of acetic acid. A: 6 ml of 170 mM hydrochloric acid before 170 mM acetic acid. B: 6 ml of 170 mM hydrochloric acid after 170 mM acetic acid. C: The hydrogen ion concentrations plotted on a semilogarithmic scale.

TABLE II. Permeability coefficients (ml · min^{-1}) and maximal chloride ion concentration decrease mM in instillations of hydrochloric acid in a cat's stomach before and after an instillation of acetic acid. The chloride ion concentration decrease is measured as the difference between the original concentration and the lowest one. Experiment no. 5 is an experiment with 170 mM HCl to which was added sodium acetate in powder form to a final concentration of 170 mM instead of 170 mM HAc.

	Permeability coefficient (ml · min^{-1})			Chloride decrease (mM)	
Exp. no.	Before HAc	After HAc	Difference %	Before HAc	After HAc
2	0.06	0.11	83	15	32
3	0.09	0.22	144	25	36
4	0.06	0.09	50	21	37
Mean value	0.07	0.14	100	20	35
5	0.09	0.13	44	26	30

can be shown that in such experiments the disappearance rate of acetic acid is less than that for hydrochloric acid, whereas in the cat's stomach the reverse is true. Note that the volume decreased in the acetic acid instillation experiments because of the hypotonicity of the solution.

B. Influence of acetic acid on subsequent instillation of hydrochloric acid.

Similar experiments as described above were performed in three consecutive steps. First 170 mM HCl was introduced and allowed to remain to diffuse for one hour (samples taken every 15 min). See Fig. 2A. In step two the same procedure was performed with 170 mM HAc, and in a third step another 170 mM HCl was instilled.

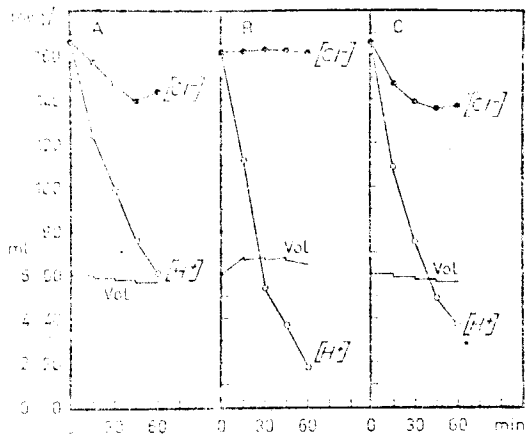

Fig. 3. An experiment with consecutive instillations of A: 6 ml of 170 mM hydrochloric acid, B: 6 ml of 170 mM hydrochloric acid to which was added sodium acetate in powder form to a final concentration of 170 mM, C: [illegible] ml of 170 mM hydrochloric acid.

See Fig. 2B. It was shown that in the second instillation of hydrochloric acid there was a higher permeability coefficient for hydrogen ions than in the first instillation. That this was not the case when the two instillations were not separated by an instillation of acetic acid, was shown in experiments where two consecutive hydrochloric acid [illegible] were analysed. In this case no increase in the permeability coefficient could be obtained. On the contrary, most of the experiments showed a decrease in the permeability coefficient for the second instillation. The results are illustrated in Fig. 2 and Table II. The permeability coefficient had increased on an average by [illegible] per cent. At the same time there appeared a significantly larger dip in the [illegible] curve after the treatment with acetic acid. It should also be [illegible] the instillation of acetic acid the stomach volume decreased markedly, as it did, to a lesser extent in the second hydrochloric acid instillation.

[illegible], no apparent increase of mucus formation was obtained.

C. Effect of a mixture of hydrochloric acid and of sodium acetate on the permeability properties for hydrochloric acid.

If sodium acetate is dissolved in hydrochloric acid the reaction

$$NaAc + HCl \rightleftharpoons NaCl + HAc$$

takes place. The pH of the solution increases because the pKa of acetic acid is higher than that of hydrochloric acid. The situation for this solution will thus be similar to that reported under B. Accordingly a persistent increase of the permeability for hydrochloric acid was obtained. An example of such an experiment is given in Table II, as experiment no. 5, and in Fig. 3. In this experiment, hypertonicity obtained because sodium acetate was added to a final solution of 170 mM, caused a volume increase.

Discussion

In non-secreting cat stomachs it was shown that the acidity decreased more rapidly when acetic acid was introduced than when hydrochloric acid was introduced. This confirms earlier results of Teorell (1939). Such an increased disappearance rate could

be due to a possible increase in vascularisation but may also be explained by a rapid permeation of the undissociated weak acid not only through the water-phase, but also through lipid membranes (Teorell 1939, Hogben *et al.* 1957, Schanker *et al.* 1957). This explanation has been applied to acetic acid as well as to many other weak acids and weak bases (Bilski and Öbrink 1959, Hogben *et al.* 1957, Milne, Scribner and Crawford 1958, Schanker *et al.* 1957).

In this paper it could, however, be shown that a second instillation of hydrochloric acid made after treatment with acetic acid resulted in a higher permeability coefficient for hydrogen ions than did the first instillation. The introduction of acetic acid thus had a persistent effect on the permeability properties of the gastric mucosa. This may be compared with the observations of Babkin and his group on the persistent effect on the secretory capabilities of the gastric mucosa after treatment with acetic acid.

During the experiments with acetic acid the "total acidity" usually decreased from 170 mM to an average of 10 mM. In this region the degree of dissociation for acetic acid varies between 0.01 and 0.04. Thus most of the acid was undissociated during the whole experiment. In the mucosal cells as well as outside of the stomach the pH is much higher (pH around 7). Under these conditions the concentration of the undissociated acetic acid is probably not more than 1 per cent; dissociation coefficient equals 0.99. The concentration of the undissociated form of acetic acid on the serosal side of the membrane can consequently be considered as being practically zero. The concentration gradient for undissociated acetic acid over the mucosal membrane will thus be directly proportional to the total concentration of acetic acid within the gastric contents.

In Fig. 1 and Fig. 2 the concentration of the hydrogen ions is plotted also on a semilogarithmic scale, showing a straight line disappearance rate for the hydrogen ions. Thus the disappearance can be treated as a diffusion following the formula

$$H = H_0 e^{-kt/p}$$

where H = the actual hydrogen ion concentration or concentration of acid at time t

H_0 = H at time t = 0

k = the permeability coefficient (ml/min)

t = time in minutes

p = the introduced volume (ml)

This formula was shown to be valid for hydrochloric acid by Teorell (1933, 1947) and Öbrink (1948). It is applicable if the actual concentration of the molecules outside the stomach is so small that it can be considered as being zero. The diffusion is looked upon as a molecular one.

Our results suggest that the acetic acid also disappears at a rate directly proportional to its concentration within the stomach.

The coupling between the probable distribution of the acetate as mentioned above, and the observed changes in the permeability properties of the gastric mucosa and its decreased secretory capabilities is, however, not yet understood. One should however consider the probable accumulation of acetate within the mucosal cells in confirmation of Martin (1963). A concomitant secretion in a diffusion experiment would give a falsely low permeability coefficient according to Öbrink and Waller (1964). In the related experiments of type B there was a mean volume increase of 0.1 ml before, but a mean decrease of 0.3 ml after the introduction of acetic acid. This difference in volume changes could possibly be explained if a hypertonicity develops in the mucosal cells due to intracellular accumulation of acetate. A small secretion at the start of the experiment cannot be excluded, however, and part of the effect of acetic acid could then be to

inhibit that secretion. The permeability coefficient for hydrochloric acid would consequently be estimated as somewhat too low in the first part of the experiment, due to the presence of hydrochloric acid secretion.

If the disappearance rate for (H^+) increases more than does the corresponding permeability for sodium ion influx, then chloride has to make a larger dip in the concentration curve than usual because of the requirements for electroneutrality. This did actually happen as can be seen in Fig. 2.

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References

BABKIN, B. P., *Secretory mechanism of the digestive glands*. 2nd ed. New York 1950.

BABKIN, B. P., C. O. HEBB and L. KRUEGER. Changes in the secretory activity of the [illegible] glands resulting from the application of acetic acid solutions to the gastric mucosa. *Q[illegible] exp. Physiol.* 1941, *31*, 63–78.

BIESKI, R. and K. J. ÖBRINK. Quantitative aspects of the elimination of neutral violet [illegible] stomach. *Acta physiol. scand.* 1959, *16*, 42–54.

GRANT, R., Calcium in gastric mucus and regulation of gastric acidity. *Amer. J. Physiol.* [illegible] *135*, 496–503.

HEINZ, E. and K. J. ÖBRINK. Acid formation and acidity control in the stomach. *Physiol.* [illegible] 1954, *34*, 645–673.

HOGBEN, C. A. M., L. S. SCHANKER, D. J. TOCCO and B. B. BRODIE. Absorbtion of drugs from the stomach. II The human. *J. Pharmacol. exp. Ther.* 1957, *120*, 540–545.

MARTIN, B. K., Accumulation of drug anions in gastric mucosal cells. *Nature* (Lond.) 1968, *[illegible]*, 986–987.

MIENS, M. D., B. H. SCRIBNER and M. A. CRAWFORD. The non-ionic diffusion and excretion of weak acids and bases. *Amer. J. Med.* 1958, *14*, 709–729.

ÖBRINK, K. J., Studies on the kinetics of the parietal secretion of the stomach. *Acta physiol. scand.* 1948, *15*, Suppl. 51.

ÖBRINK, K. J., and M. WALLER. The transmucosal migration of water and hydrogen ions in the stomach. 1964. In press.

SCHANKER, L. S., P. A. SHORE, B. B. BRODIE and C. A. M. HOGBEN. Absorbtion of drugs from the stomach. I The rat. *J. Pharmacol. exp. Ther.* 1957, *120*, 528–539.

SHORE, P. A., B. B. BRODIE and C. A. M. HOGBEN. The gastric secretion of drugs: a pH partition hypothesis. *J. Pharmacol. exp. Ther.* 1957, *119*, 361–369.

TEORELL, T., Untersuchungen über die Magensaftsekretion. *Skand. Arch. Physiol.* 1933, *66*, 225–318.

TEORELL, T., On the permeability of the stomach mucosa for acids and some other substances. *J. gen. Physiol.* 1939, *25*, 263–274.

TEORELL, T., Electrolyte diffusion in relation to the acidity regulation of the gastric juice. *Gastroenterology* 1947, *9*, 425–443.

Italian Translation

Acetic Acid in the Lymph and Blood with Respect to Intestinal Absorption and Tissue Metabolism*

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Source: Arch. sc. biol. 36. 335-39, May-June 1952.

Introduction

Until recently, accurate determinations of acetic acid in circulating fluids of man and laboratory animals did not exist, since the only literature data (the most recent and precise data were obtained by Reid (1)) refer exclusively to ruminants, which from this viewpoint represent a special case, in view of the particular type of digestion of such animals.

Recently, Ciaranfi and Fonnesu (2) have proposed a microphotometric method for acetic acid and have determined this compound in human blood and in organs of some laboratory animals, and were able to establish that acetic acid is a normal component of blood and tissues.

In comparative studies on human arterial and venous blood, Fonnesu (3) has shown that the concentration of acetic acid is higher in arterial blood than in venous blood, and has also noted that this concentration undergoes a characteristic modification during the digestive period. This dependence of the acetic acid content in peripheral blood on digestion leads us to suspect 2 possibilities, namely: 1) that acetic acid comes (originates) directly from the intestine and 2) that it is formed in the liver by a shortening of more complex carbon chains, which certainly include higher fatty acids, supplied to this organ by the intestine.

* This study was carried out with the aid of a grant from the High Commission for Hygiene and Public Health.

Such hypotheses obviously refer only to the digestive increment of the blood acetate content, since the formation of this compound in the intermediate metabolism of organs is an undisputed fact; this process tends to maintain a constant concentration of this acid in the blood during the non-digestive period.

In order to verify these hypotheses, acetic acid determinations were carried out in the lymph of the thoracic duct and in blood taken from various areas of the circulatory system of the dog during the digestive period. This research is the object of the present study.

Materials and Techniques

The test animals used were normal adult dogs of uniform height and medium size, in good nutritional condition. After about 10 hours of fasting, the animals were given a food mixture consisting of 400 g bread, 100 g milk, 70 g non-rancid lard, 150 g meat and water *ad libitum*.

During the digestive period (45 min after food intake), the animals were anesthetized with an intravenous injection of 2.5% sodium pentothal, without previous basic anesthesia, and lymph and blood were collected as rapidly as possible. Lymph was collected from the thoracic duct as follows.

With the animal's head slightly extended, a vertical incision was made on the neck, after previous shaving, at a distance of 1 cm to the left of the median line and parallel to it, extending from the level of the thyroid cartilage down to 1-2 cm below the sternal fork. After cutting the soft tissues and separating the ventral muscles, the periesophageal connective tissue was reached and cut away, then, proceeding cautiously by the blunting route, the thoracic duct was reached, which crosses the esophagus and trachea

on the left side, attempting to isolate it in the vicinity of its outlet (starting point of the left brachiocephalic vein or end adjacent to the vena subclavia).

The thoracic duct has the appearance of a fine milk-white ribbon, occasionally bifurcated, of nonuniform diameter due to varicose-like dilatations.

Since the animal was sacrificed at the end of the operation, it was found convenient to perform a more extensive incision by also cutting the pectoral muscles in order to make the isolation of the thoracic duct more easy.

After closing the duct with pincers at a distance of about 1 cm from its outlet, lymph was collected by means of a syringe with a needle of medium diameter. The collection was done by small successive aspirations, making use of the rhythmic fillings of the duct corresponding to respiratory movements.

The amount of lymph used on one determination was 5 ml, but it was possible, in most cases, to collect a larger quantity in order to obtain a couple of samples.

Making use of the surgical incision, 5 ml of blood was then taken from the inner jugular vein, using a syringe equipped with a fine needle. Immediately afterwards, a long abdominal cut was made along the white line and, always by means of the syringe, further 5 ml-samples of blood were collected from the aorta, the candal vena cava at its point of origin, from the renal vein, the portal vein and the suprahepatic veins, after previous ligature of the hepatic artery, and from the caudal vena cava immediately below the liver. Finally, an equal blood sample was taken from the right ventricle.

The determination of acetic acid in the lymph and blood samples was done by the above-mentioned photometric method (Ciaranfi and Fonnesu), making the lymph slightly alkaline as was described for tissues.

Results

The results are given in Table 1.

Table 1

Determination of acetic acid in the lymph and blood of normal dogs during the digestive period

N. dell'animale	Acido acetico (mg p. 100 ml)							
	linfa del dotto toracico	Aorta	V. cava caudale (origine)	V. renale	V. porta	VV. sovraepatiche	V. giugulare interna	cuore destro
1		9,0	7,8		7,2	5,0		
2		6,8	4,2		10,4	9,0		
3		6,8	6,8		9,2	6,8		
4		10,8	9,0		11,0	9,8		
5	15,5	11,4					10,6	
6	23,0	6,4					6,0	
7	15,0	10,8					9,8	
8	18,0	9,8	4,0		6,0			
9	20,0	6,4	6,0		8,0			
10	19,0	10,2	6,0		9,8			
11		13,0	10,0	8,0	18,0	15,0	5,8	13,0
12		7,0	5,6	5,8	7,2	9,0	5,6	6,4
13		9,8	9,6	8,4	8,5	8,6	9,6	9,2
medie	*18,5*	*9,0*	*7,0*	*7,5*	*9,5*	*9,0*	*8,0*	*9,5*

1 -- Thoracic duct lymph
2 -- Suprahepatic veins
3 -- Inner jugular vein
4 -- Right heart

Discussion and Conclusions

An examination of the data listed in Table 1 makes it possible to answer the question concerning the origin of the digestive fraction of blood acetic acid.

From a comparison of average acetate concentrations in the blood of the portal vein and in the blood of suprahepatic veins, it is evident that the liver does not participate in the digestive increment of the acetic acid concentration. Naturally, this does not mean that this organ is incapable of producing acetic acid, but only that the balance between acetic acid produced and acetic acid converted into other compounds or destroyed is practically equal. In addition, the hypothesis that intestinal absorption of acetic acid takes place primarily by the venous route can be discarded, since the acetate concentration in portal blood, although higher than that in venous blood flowing in from other organs and tissues, is almost equal to the concentration in arterial blood.

The high acetic acid content in the lymph of the thoracic duct shows that the transport of this compound from the intestinal wall to the blood circulation system takes place, at least to a major part, by the lymphatic route. Such a mode of transport explains the higher acetate content in the blood of the right heart area, as compared to the content in the caudal vena cava and the inner jugular vein, and also explains why the venous blood of the right heart has almost the same acetate content as arterial blood; this latter fact makes it possible to exclude that significant modifications of the acetic acid content take place through the small circle.

In view of the statement by Eckstein (4) that general anesthesia reduces the entity of the intestinal absorption of fats in dogs, it is probable

that, in non-anesthetized animals, the acetate ratio in the lymph of the thoracic duct is even greater. However, this does not concern our case, because our problem here was to clarify the modality of the digestive increment of acetate, and not to establish a balance (ratio) between ingested foods and absorbed acetic aicd.

It now remains to establish whether acetic acid derived from the intestine originates as such from the lumen, or whether it is rather produced at the level of the intestinal wall by probable β -oxidation of absorbed higher fatty acids.

The first hypothesis appears to be unlikely since digestive enzymes capable of forming acetate have not been noted, while the bacterial flora is absent or too scarce in the lumen of the small intestinal loops to be able to participate in such a process. The intestinal production of acetic acid by Escherichia coli can probably take place in the large intestine, since this substance is one of the end products of the complex fermentation of glycids effected by this microorganism. Therefore, a direct absorption of acetate from the lumen of the large intestine, as a result of a secondary digestion process, could take place, but it could not be responsible for the digestive increment of blood acetic acid, which is noted a short time after food intake and which disappears rather rapidly, as was shown by Fonnesu (3) in man. In fact, about 4-5 hours after food intake, the acetate concentration in the blood is again equal to its original value.

The second hypothesis, namely acetic acid production at the level of the intestinal wall, no doubt appears the most probable one and is the object of studies which will be published shortly.

Summary

Determinations of acetic acid in the lymph of the thoracic duct and in blood taken from various sections of the circulatory system of dogs during the digestion period have shown the following: 1) that, at least to a major-extent, the digestive increment of acetic acid is due to intestinal absorption by the lymphatic route; 2) that the concentration of acetic acid in portal blood is almost equal to that of arterial blood; 3) that the liver does not intervene in the determination of the digestive increment of acetate, since the concentration of this product is approximately the same in portal blood and in the blood of adrenal veins; 4) that the difference between acetic acid concentration in arterial blood and peripheral venous blood is due to two factors: on the one hand, the supply of acetate through the thoracic duct, and on the other, the peripheral consumption of acetate in tissues.

Bibliography

(1) Reid R. L. - *Nature*, 1950, *165*, 448.
(2) Ciaranfi E. a. A. Fonnesu - *Biochem. J.*, 1952, *50*, 698.
(3) Fonnesu A. - *Bull. Soc. Chim. biol.*, 1951, *33*, 1021.
(4) Eckstein H. C. - *J. biol. Chem.*, 1925, *62*, 743.

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L'ACIDO ACETICO NELLA LINFA E NEL SANGUE IN RAPPORTO ALL'ASSORBIMENTO INTESTINALE E AL METABOLISMO TESSUTALE [1]. Di A. Fonnesu ed E. Ciaranfi.

Dall'Istituto di Patologia generale dell'Università di Perugia

Premesse

Fino a poco tempo fa non esistevano determinazioni precise di acido acetico negli umori circolanti dell'Uomo e degli animali da laboratorio perché gli unici dati della letteratura, i più recenti e precisi dei quali si debbono al Reid (1), si riferiscono esclusivamente ai Ruminanti che rappresentano da questo punto di vista un caso particolare, considerato il tipo peculiare di digestione di questi animali.

Recentemente Ciaranfi e Fonnesu (2) hanno proposto un micrometodo fotometrico per l'acido acetico ed hanno determinato questa sostanza nel sangue umano e nel sangue e negli organi di alcuni animali da laboratorio, riuscendo a stabilire che l'acido acetico è un componente normale del sangue e dei tessuti.

In ricerche comparative tra sangue arterioso e venoso umano, Fonnesu (3) ha dimostrato che la concentrazione di acido acetico è maggiore nel sangue arterioso che nel venoso, osservando inoltre che essa subisce una caratteristica modificazione in periodo digestivo. Questa dipendenza dalla digestione del tasso di acido acetico nel sangue periferico lasciava supporre due possibilità e cioè: 1) che l'acido acetico provenisse direttamente dall'intestino; 2) che si formasse nel fegato per accorciamento di catene carboniose più complesse, tra le quali certamente erano acidi grassi superiori, veicolate a quest'organo dall'intestino.

Tali ipotesi si riferiscono ovviamente soltanto all'incremento digestivo del tasso ematico di acetato poiché è indiscussa la formazione di questa sostanza nel metabolismo intermedio degli organi, processo

[1]) La presente ricerca è stata eseguita mediante una sovvenzione dell'Alto Commissariato per l'Igiene e la Sanità Pubblica.

che tende a mantenere costante la concentrazione di questo acido nel sangue durante il periodo non digestivo.

Allo scopo di verificare queste ipotesi si sono eseguiti dei dosaggi di acido acetico nella linfa del dotto toracico e nel sangue prelevato da diverse sezioni del circolo di Cane normale in periodo digestivo. Questa ricerca è l'oggetto del presente lavoro.

Materiale e tecnica

Come animali da esperimento si sono usati Cani adulti normali, di taglia uniforme e di media grandezza, in buone condizioni di nutrizione. Dopo circa 10 h di digiuno si somministrava agli animali un pasto costituito da pane g 400, latte g 100, lardo (non rancido) g 70, carne g 150 ed acqua a volontà.

In periodo digestivo (45 min dal pasto) l'animale veniva anestetizzato con pentothal sodico al 2,5 p. 100 per via intravenosa, senza previa anestesia di base, usando la quantità minima sufficiente per una buona anestesia, e si procedeva, il più rapidamente possibile, ai prelievi di linfa e di sangue.

Per il prelievo della linfa dal dotto toracico si procedeva come segue.

A testa leggermente estesa si praticava sul collo, previa rasatura della parte, un'incisione verticale 1 cm circa a sinistra della linea mediana e parallela a questa, che dall'altezza della cartilagine tiroide arrivasse in basso 1-2 cm al di sotto della forchetta sternale. Incisi i tessuti molli e divaricati i muscoli ventrali, si raggiungeva e si scollava il connettivo periesofageo e, procedendo cautamente per via smussa, si arrivava al dotto toracico, che incrocia sul lato sinistro esofago e trachea, cercando di isolarlo fino in prossimità del suo sbocco (inizio della vena brachiocefalica sinistra o estremo prossimale della vena succlavia).

Il dotto si presenta come un cordoncino di color bianco-latte, talvolta biforcato, di calibro non uniforme per dilatazioni di aspetto varicoso.

Poiché l'animale alla fine dell'intervento veniva sacrificato, si è trovato conveniente praticare una breccia più ampia procedendo anche al taglio dei muscoli pettorali allo scopo di rendere più agevole l'individuazione del dotto toracico.

Chiuso il dotto con una pinza ad 1 cm circa dal suo sbocco, la linfa veniva prelevata mediante siringa munita di un ago di medio calibro. Il prelievo veniva fatto con piccole aspirazioni successive approfittando dei riempimenti ritmici del dotto corrispondenti ai movimenti respiratori.

La quantità di linfa usata per una determinazione era di 5 ml ma è stato possibile, nella maggioranza dei casi, prelevarne una quantità maggiore in modo da consentire prove in coppia.

Approfittando della breccia chirurgica, si prendevano poi 5 ml di sangue dalla vena giugulare interna, servendosi di una siringa munita di ago sottile. Subito dopo si praticava un lungo taglio addominale lungo la linea alba e, sempre mediante siringa, si aspiravano altri campioni di 5 ml di sangue rispettivamente dall'aorta, dalla vena cava caudale alla sua origine, dalla vena renale, dalla vena porta e dalle vene sovraepatiche, previa legatura dell'arteria epatica, e della vena cava caudale subito al di sotto del fegato. In ultimo, si aspirava un ugual campione di sangue dal ventricolo destro.

Il dosaggio dell'acido acetico nei campioni di linfa e di sangue veniva eseguito col metodo fotometrico sopracitato (Ciaranfi e Fonnesu, 2), alcalinizzando leggermente la linfa come è descritto per i tessuti.

Risultati

I risultati sono riportati nella tabella I.

Tabella I.

Dosaggi di acido acetico nella linfa e nel sangue di Cane normale in periodo digestivo.

N. dell'animale	Acido acetico (mg p. 100 ml)							
	linfa del dotto toracico	Aorta	V. cava caudale (origine)	V. renale	V. porta	VV. sovraepatiche	V. giugulare interna	cuore destro
1		9,0	7,8		7,2	5,0		
2		6,8	4,2		10,4	9,0		
3		6,8	6,8		9,2	6,8		
4		10,8	9,0		11,0	9,8		
5	15,5	11,4					10,6	
6	23,0	6,4					6,0	
7	15,0	10,8					9,8	
8	18,0	9,8	4,0		6,0			
9	20,0	6,4	6,0		8,0			
10	19,0	10,2	6,0		9,8			
11		13,0	10,0	8,0	18,0	15,0	5,8	13,0
12		7,0	5,6	5,8	7,2	9,0	5,6	6,4
13		9,8	9,6	8,4	8,5	8,6	9,6	9,2
medie	*18,5*	*9,0*	*7,0*	*7,5*	*9,5*	*9,0*	*8,0*	*9,5*

Discussione e conclusioni

L'esame dei dati riportati nella tabella I permette di rispondere ai quesiti posti circa la provenienza della frazione digestiva dell'acido acetico ematico.

Dal confronto tra le medie delle concentrazioni di acetato nel sangue della vena porta e in quello delle vene sovraepatiche risulta evidente che il fegato non partecipa all'incremento digestivo del tasso di acido acetico. Ciò naturalmente non significa che tale organo non sia capace di produrre acido acetico ma solo che il bilancio tra acido acetico prodotto e acido acetico trasformato in altri composti o demolito è praticamente in pareggio. Oltre a ciò, si può scartare l'ipotesi che l'assorbimento intestinale dell'acido acetico avvenga prevalentemente per via venosa poiché la concentrazione di acetato nel sangue portale, pur essendo più elevata di quella nel sangue venoso refluo da altri organi e tessuti, risulta pressochè uguale a quella nel sangue arterioso.

L'elevato contenuto in acido acetico della linfa del dotto toracico dimostra che il trasporto di questa sostanza dalla parete intestinale al circolo sanguigno avviene, almeno per la maggior parte, attraverso la via linfatica. Tale modalità di trasporto dà ragione del più alto contenuto in acetato del sangue del cuore destro in confronto a quello della vena cava caudale e della vena giugulare interna e spiega perché il sangue venoso del cuore destro abbia pressochè lo stesso tenore in acetato del sangue arterioso, fatto questo che ci permette di escludere che modificazioni sensibili del tasso di acido acetico si verifichino attraverso il piccolo circolo.

Poiché è stato affermato da Eckstein (4) che l'anestesia generale diminuisce l'entità dell'assorbimento intestinale dei grassi nel Cane, è probabile che in animali non anestetizzati il tasso di acetato nella linfa del dotto toracico sia ancora maggiore. Ciò tuttavia non riguarda il nostro caso perché il problema propostoci era quello di chiarire le modalità di incremento digestivo dell'acetato e non quello di stabilire un bilancio tra alimenti ingeriti ed acido acetico assorbito.

Resta ora da precisare se l'acido acetico di derivazione intestinale provenga come tale dal lume o venga piuttosto prodotto a livello della parete intestinale per probabile β-ossidazione di acidi grassi superiori assorbiti.

La prima ipotesi appare poco verosimile perché non sono noti fermenti digestivi capaci di formare acetato, mentre la flora batterica è assente o troppo scarsa nel lume del tenue per poter prendere sensibile parte ad un processo del genere. Una produzione intestinale di acido acetico da parte dell'*Escherichia coli* si verifica probabilmente

nel crasso, poiché tale sostanza è uno dei termini della fermentazione complessa dei glicidi operata da questo microrganismo. Pertanto un assorbimento diretto di acetato dal lume del crasso, in seguito ad un processo di digestione secondaria, si potrebbe verificare ma esso non potrebbe essere responsabile dell'incremento digestivo dell'acido acetico ematico che si verifica a breve distanza dall'assunzione del cibo e che abbastanza rapidamente si annulla, come è stato dimostrato da Fonnesu (3) nell'Uomo. Infatti a circa 4-5 h dal pasto la concentrazione di acetato nel sangue è di nuovo al valore di partenza.

La seconda ipotesi, cioè la produzione di acido acetico a livello della parete intestinale, appare indubbiamente la più probabile ed è oggetto di indagini che verranno successivamente rese note.

R i a s s u n t o . Dosaggi di acido acetico eseguiti su linfa del dotto toracico e sangue prelevato da varie sezioni del circolo di Cani normali in periodo digestivo hanno messo in evidenza: 1) che almeno in fortissima prevalenza l'incremento digestivo dell'acido acetico è dovuto ad assorbimento intestinale per via linfatica; 2) che l'acido acetico nel sangue portale è in concentrazione pressochè uguale che nel sangue arterioso; 3) che il fegato non interviene nel determinare l'incremento digestivo di acetato in quanto la concentrazione di questa sostanza è all'incirca uguale nel sangue portale e in quello delle vene sovraepatiche; 4) che la differenza di concentrazione di acido acetico tra sangue arterioso e venoso periferico è dovuta a due fattori: da un lato l'apporto di acetato attraverso il dotto toracico, dall'altro il consumo periferico di acetato nei tessuti.

BIBLIOGRAFIA

(1) Reid R. L. - *Nature*, 1950, *165*, 448.
(2) Ciaranfi E. a. A. Fonnesu - *Biochem. J.*, 1952, *50*, 698.
(3) Fonnesu A. - *Bull. Soc. Chim. biol.*, 1951, *33*, 1021.
(4) Eckstein H. C. - *J. biol. Chem.*, 1925, *62*, 743.

Pervenuto in Redazione il 1° di agosto del 1951.

Food and Agriculture Organization of the United Nations 1970
A review of the technological efficacy of some antimicrobial agents
FAO Nutr. Meetings Rep. Ser. 48C, WHO Tech. Rep. Ser. 462, 14th Report ECFA
p. 25

Food Protection Committee, Food and Nutrition Board 1965
Chemicals Used in Food Processing
National Academy of Sciences, National Research Council,
Washington, D.C.
Publ. No. 1274: pp. 3, 41, 66

The metabolism of glucose, acetate, palmitate, stearate and oleate in pigs

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1. Isotope dilution techniques were used to measure the rates of entry and oxidation of glucose, acetate, palmitate, stearate and oleate in fed and fasted (16 h) pigs.

2. Glucose entry rates measured in eight fed and five fasted (16 h) pigs were 20·6 and 8·0 mg/min per $kg^{0.75}$ (mean values) respectively. The entry rates in fed pigs were lower than the calculated rates of glucose uptake from the alimentary tract, and the validity of glucose entry rates determined under these conditions is discussed.

3. Acetate entry rates were unrelated to the fibre content of the diet, and acetate produced from endogenous metabolism was the main contributor to total acetate production.

4. Increased rates of entry and oxidation of palmitate, stearate and oleate were observed in fasted pigs.

5. Glucose production in fasted pigs was inhibited by raising the concentration of blood glucose. Infusion of insulin in the fed pig caused a decrease in glucose production and a simultaneous reduction in the mobilization of free fatty acids. When blood glucose concentrations fell below about 55 mg/100 ml the release of free fatty acid into the circulation was restored.

There have been few quantitative studies on glucose and fatty acid metabolism in pigs. Glucose tolerance tests in mature pigs gave results which closely resembled those obtained in man (Eveleth & Eveleth, 1935; Hanawalt, Link & Sampson, 1947), and the responsiveness of pigs to insulin was also similar to that shown by man (Sampson & Graham, 1943). Acetate is normally present in the caecum and large intestine of pigs (Elsden, Hitchcock, Marshall & Phillipson, 1945–6; Friend, Nicholson & Cunningham, 1963), and the absorption of acetate from these sites was demonstrated by Barcroft, McAnally & Phillipson (1944). Friend, Nicholson & Cunningham (1964) compared acetate levels in arterial and portal blood samples from anaesthetized pigs, and concluded that acetate absorbed from the alimentary tract made a quantitatively significant contribution to the energy requirement of the pig. In the present studies we have used isotope dilution techniques to measure the rates of turn-over of glucose, acetate, palmitate, stearate and oleate in unanaesthetized fed and starved (16 h) pigs. The effects of raised blood concentrations of glucose on glucose production, and the effects of insulin on glucose and plasma free fatty acid (FFA) metabolism, were also studied.

MATERIALS AND METHODS

Experimental animals

Large White × Wessex pigs (6–12 weeks of age) were housed in metabolism crates in the laboratory. At least 3 d before an experiment polyethylene catheters (Portex Plastics Ltd, UK) were inserted under general anaesthesia into the external jugular vein (1·7 mm external diameter) and the carotid artery (1·52 mm ED) and in some cases

the external thoracic vein (1·52 mm ED). The catheters were kept patent for periods of up to 1 month by twice-daily flushing with heparin–saline (2500 units/ml).

Diet

Pigs were normally fed *ad lib.* on a pelleted diet (22 % protein, 6·5 % fat, 2 % fibre). During some of the experiments, which are indicated below, the fibre content was increased to 5–6 % by the addition of cellophane flake. Water was available at all times. Food and water intakes were recorded.

Experimental procedure

The intravenous infusions of isotopes were carried out in the animals' own crates, using procedures similar to those described earlier (Annison & White, 1961). Rates of entry of substrate into the total body pool were calculated as described earlier (Annison & White, 1962). In all experiments, unless otherwise indicated, the substrate was infused for a period of 4 h, and blood samples were withdrawn during the final hour of the infusion. It was our experience that this period of infusion (3 h) was necessary for the specific radioactivity (sra) of the metabolites to reach constant levels; in those cases where the plasma levels or the sra of the metabolites varied by more than ± 10 %, the experiment was rejected. The sra of CO_2 in arterial blood during the terminal stages of the infusion in relation to the sra of the infused metabolite provided a rough measure of the contribution of the substrate to total CO_2 production (Annison, Brown, Leng, Lindsay & West, 1967). Details of the infusions of the different substrates, and the methods of sampling are described below.

Metabolism of glucose. Uniformly labelled [^{14}C]glucose in 0·9 % (w/v) NaCl (0·3 μCi; 1 mg/ml) was infused at 0·5 ml/min into the jugular vein immediately after a priming dose (15 μCi) of labelled glucose. Blood samples (8 ml) were withdrawn at 10 min intervals from the carotid artery during the last hour of the infusion. In three experiments the [^{14}C]glucose was infused into the external thoracic vein and five pairs of samples of carotid and jugular blood were taken simultaneously. In one experiment to examine the effect of glucose load on glucose metabolism, unlabelled glucose was infused with labelled glucose at rates varying from 50 to 200 mg/min.

Metabolism of acetate. Sodium[1,2-^{14}C]acetate in 0·9 % (w/v) NaCl was infused (0·5 μCi; 1 mg/min) into the jugular vein without a priming dose. Blood samples (10 ml) were withdrawn from the carotid artery as described above.

Metabolism of plasma palmitate, stearate and oleate. [U-^{14}C]palmitic, [U-^{14}C]stearic or [U-^{14}C]oleic acid, and [9,10-^{3}H]palmitic acid were infused as aqueous solutions of their sodium salts in albumin, prepared as described by Laurell (1957), at the rate of 0·25 μCi/min (except [9,10-^{3}H]palmitate; see below) into the jugular vein. Albumin (bovine serum albumin, fraction V powder), obtained from Sigma Chemical Co. (London) Ltd, was used. Arterial blood samples (10 ml) were taken as described above.

Effect of insulin on the metabolism of glucose and palmitate

[U-^{14}C]glucose and [9,10-^{3}H]palmitate were simultaneously infused into the jugular vein of a fed pig (29 kg) at the rate of 0·257 μCi and 1·420 μCi/min respectively, with a priming dose of 25 μCi of [U-^{14}C]glucose given prior to the start of the infusion. From the 260th min of the infusion, insulin (Insulin BP; Boots Pure Drug Co. Ltd) was infused into the external thoracic vein at the rate of 2 m-units/min per kg body-weight, and was stopped 120 min later. The dual isotope infusion continued for 415 min.

Radioactive materials

Labelled compounds were obtained from the Radiochemical Centre, Amersham.

Chemical methods

Plasma glucose sra. Plasma glucose concentrations were measured with glucose oxidase (Huggett & Nixon, 1957). Glucose was isolated as the pentaacetate (Jones, 1965) after purification with ion-exchange resins. Preliminary work showed that labelled acetate and labelled lactate may both contaminate glucose pentaacetate if present in the reaction mixture. Plasma (1 ml) containing carrier glucose (150 mg) was deproteinized by adding H_2O (4 ml), 0·3 N-$Ba(OH)_2$ (2 ml) and, after shaking, 2 ml of $ZnSO_4.7H_2O$ (5% w/v). The filtrate was passed through a column (diam. 1·5 cm) of a mixed bed of Amberlite (British Drug Houses Ltd) ion-exchange resins (IRC 50 and IR45, 2:1, v/v), and the glucose eluted with 100 ml of deionized water. The eluate was concentrated in vacuo before preparation of the pentaacetate, which was assayed for radioactivity as described below.

Blood acetate sra. The concentration of acetate in blood was measured by gas-liquid chromatography, using an internal standard. Sodium propionate (100 μg) was added to blood (5 ml) and the total volatile fatty acids (VFA) were isolated by deproteinization and steam distillation as described earlier (Annison & White, 1962). The neutralized distillate, containing 50% excess NaOH, was concentrated to 2–3 ml by boiling. A sample (10%) was dried down, the sodium salts were dissolved in 100 μl of 5% ortho-phosphoric acid, and 1–5 μl were transferred to a Model 104 gas chromatograph (Pye Unicam Ltd, Cambridge) fitted with a flame ionization detector. The stationary phase was acid-washed Chromosorb W coated with 10% (w/w) neo-pentyl glycol adipate (Pye Ltd, Cambridge), and the operating temperature was 125°, with nitrogen as carrier gas. The relative areas of the acetate and propionate peaks (corrected for detector response) allowed calculation of the concentration of acetate in the original blood sample.

The remainder of the VFA sample was assayed for radioactivity as described below.

Blood lactate sra. Blood lactate concentrations were measured enzymically (Barker & Britton, 1957). Lactate was isolated from blood (5 ml) as described earlier (Annison, Lindsay & White, 1963), using 100 mg of lithium lactate as carrier. The isolated zinc lactate was assayed for radioactivity as described below.

Plasma free fatty acids (FFA) sra. Plasma lipids were extracted by the method of Folch, Lees & Stanley (1957), and an internal standard, heptadecanoic acid, was

added to the extract in the proportion 20 μg/ml plasma. The FFA fraction was isolated by thin-layer chromatography on silica gel in the system diethyl ether–hexane–acetic acid (30:70:2, v/v) and eluted from the silica with 15 ml of diethyl ether–light petroleum (b.p. 40–60°)–formic acid (50:50:1, v/v). The concentration of individual fatty acids in the FFA fraction was determined by gas-liquid chromatography using the method described by West & Rowbotham (1967). A portion of the separated FFA fraction was assayed for radioactivity as described below.

Blood carbon dioxide sra. Blood CO_2 was isolated as described by Annison & Lindsay (1961) and assayed for radioactivity as described below.

Assay of radioactivity. All substrates were assayed by liquid scintillation counting using a Packard 4000 Series Spectrometer. Corrections for quenching were made using an external standard of ^{226}Ra. Fatty acids and glucose pentaacetate were assayed in solution in toluene containing 0·4%, 2,4-diphenyloxazole (PPO). Finely divided $BaCO_3$ and zinc lactate were assayed in suspension in a gel of low-density silica (Cab-O-Sil; Packard Co., Wembley, Middlesex) 3·4% w/v, in toluene containing 0·4% PPO. Acetate samples were dissolved in water (1 ml) and mixed with 15 ml of a mixture (2:1, v/v) of toluene containing 0·4% PPO and a non-ionic detergent (Triton-X-100; Lennig Chemical Co., Jarrow-on-Tyne) which gave a one-phase system suitable for counting.

RESULTS

Equilibrium of bicarbonate pools

The sra of arterial blood CO_2 during the infusion of ^{14}C-labelled glucose or acetate became roughly constant after 150–180 min (Table 1). In most instances the values rose by 5–10% during the period 180–240 min, but insufficient values were obtained to allow calculation of a terminal asymptotic value (Annison *et al.* 1967).

Metabolism of glucose

Mean arterial plasma glucose levels in seven fed and six fasted (16 h) pigs were 111 (range 101–119) and 96 (87–110) mg/100 ml respectively (Table 2). In three experiments the mean arteriovenous differences in plasma glucose across the head were 9, 7 and 5 mg/100 ml. The oxidation of glucose by the tissues of the head was indicated by the increased specific radioactivity of jugular blood $^{14}CO_2$ relative to arterial $^{14}CO_2$ (Table 1).

Glucose entry rates measured in seven fed and six fasted (16 h) pigs were 20·6 (SE 1·41) and 8·0 (SE 0·59) mg/min per kg$^{0.75}$ respectively (Table 2). The mean value in fed pigs was considerably lower than the rate of uptake of glucose from the alimentary tract (26–32 mg/min per kg$^{0.75}$) calculated from the known intakes of starch, sucrose and lactose. The rates of intake of the glucose sources were based on the mean daily consumption of food recorded over a period of about 7 d prior to the infusion. In estimating the uptake of glucose, complete hydrolysis and absorption were assumed and galactose and fructose derived from lactose and sucrose respectively were calculated as glucose.

The contri[illegible]ns of glucose to total CO_2 production were 31 % (SE 5·7) and 16% (SE 1·9) respectively in fed and starved pigs (Table 2).

Table 1. *Changes in the specific radioactivity of $^{14}CO_2$ and the glucose concentrations in carotid and jugular blood during the infusion of [U-^{14}C]glucose*

(The values are the results of a typical experiment on one pig)

Time (min)	Plasma glucose (mg/100 ml) Carotid	Plasma glucose (mg/100 ml) Jugular	Specific radioactivity of blood $^{14}CO_2$ (μCi/g C) Carotid	Specific radioactivity of blood $^{14}CO_2$ (μCi/g C) Jugular
60	—	—	1·85	—
120	—	—	1·85	—
140	—	—	2·30	—
160	—	—	2·36	—
180	98	94	2·38	2·40
195	103	96	2·33	2·45
210	103	98	2·30	2·40
225	102	95	2·32	2·39
240	101	96	2·39	—

Table 2. *Levels of plasma glucose, rates of entry of glucose, and the contribution of glucose to total CO_2 production in fed and fasted (16 h) pigs*

Nutritional status	Plasma glucose (mg/100 ml)	Entry rate (mg/min kg$^{0·75}$)	Contribution to total CO_2 output (%)
Fed	112	22·0	49
	119	16·9	41
	105	19·1	25
	101	22·8	12
	115	22·0	20
	115	26·0	37
	110	15·2	—
General mean ± SE	111 ± 2·36	20·6 ± 1·41	31 ± 5·7
Fasted	100	5·6	16
	97	9·5	17
	96	8·6	24
	87	7·0	11
	110	8·6	18
	89	8·8	12
General mean ± SE	96 ± 3·69	8·0 ± 0·59	16 ± 1·9

Glucose entry rates were correlated with plasma level ($r = 0·671$; $P < 0·02$) and the contribution of glucose to total CO_2 production showed some dependence on substrate concentration ($r = 0·719$; $P < 0·01$).

Effect of glucose load

When unlabelled glucose was infused with labelled glucose at a rate (200 mg/min) sufficient to raise the circulating level of glucose to 140–150 mg/100 ml in the starved (24 h) pig, glucose production was completely inhibited (Table 3). At lower plasma

concentrations (120–150 mg/100 ml) achieved by infusing glucose a[illegible] 50 mg/min, partial inhibition was indicated by the low entry rates obtained, after correction for the input of carrier glucose.

Metabolism of acetate

Mean arterial blood levels of acetate in fourteen fed and four fasted pigs were 2·5 (SE 0·10) and 2·2 (SE 0·13) mg/100 ml respectively. The mean rates of entry of acetate in six fed and four fasted pigs were 11·8 and 9·2 mg/min per kg$^{0·75}$ respectively, and

Table 3. *Inhibition of entry of endogenous glucose at raised concentrations of plasma glucose in the pig*

Expt no.	Plasma glucose (mg/100 ml)	Rate of infusion of unlabelled glucose (A) (mg/min)	Total entry rate of glucose (B) (mg/min)	Entry rate of endogenous glucose (B–A) (mg/min)
1	120	50	129	79
2	126	100	151	51
3	136	150	173	23
4	141	200	204	4
5	150	200	207	7

Table 4. *Levels of blood acetate, rates of entry of acetate, and the contribution of acetate to total CO_2 production, in fed and fasted (16 h) pigs given low-fibre (L) and high-fibre (H) diets*

Nutritional status	Level of dietary fibre	Blood acetate (mg/100 ml)	Entry rate (mg/min kg$^{0·75}$)	Contribution to total CO_2 output (%)
Fed	L	2·5	12·1	12
	L	2·2	11·9	12
	L	2·4	8·1	9
	H	2·8	12·9	10
	H	2·3	11·1	10
	H	2·5	14·6	18
General mean ± SE		2·5 ± 0·10	11·8 ± 0·88	12 ± 1·46
Fasted	L	2·4	8·8	22
	H	1·9	8·2	18
	H	2·1	11·0	24
	H	2·3	8·7	22
General mean ± SE		2·2 ± 0·13	9·2 ± 0·71	21 ± 1·49

corresponding values for the contribution of acetate to total CO_2 output were about 12% and 21% (Table 4).

The fibre content of the diets had no influence on either the concentrations of blood acetate, or the measured entry rates of acetate.

Metabolism of plasma palmitate, stearate and oleate

The composition of the plasma FFA fraction and the concentrations of individual plasma FFA were collated from the many analyses made during the course of experiments on a total of nine experimental animals, which were either fed or fasted

(Table 5). There were significant shifts in the composition of the plasma FFA on fasting, the proportion of fatty acids (C_{12}, C_{14}, C_{15}) of medium chain length and of linoleate (18:2) decreasing and the proportion of oleate (18:1) increasing markedly. Although in general the levels of individual FFA were higher in fasted than in fed pigs, the variability in absolute concentrations between animals was large and for any fatty acid the difference between the mean concentration in fed and fasted pigs was not statistically significant.

Table 5. *Fatty acid composition (major components as percentage by weight of the total) of plasma free fatty acids, and plasma levels of individual fatty acids in fed and fasted (16 h) pigs*

(Values for composition are means and standard deviations; values for concentration are means with their ranges)

	Fed		Fasted	
Fatty acid	Composition (%)	Concentration (mg/100 ml)	Composition (%)	Concentration (mg/100 ml)
12:0	2·14 ± 0·67	0·30 (0·24–0·42)	0·61 ± 0·29	0·34 (0·09–0·94)
14:0	3·43 ± 1·34	0·31 (0·16–0·60)	1·72 ± 0·44	0·58 (0·19–1·45)
15:0	2·27 ± 1·22	0·21 (0·08–0·39)	1·01 ± 0·61	0·38 (0·09–0·93)
16:0	27·91 ± 5·57	2·26 (1·26–3·80)	26·28 ± 7·38	8·25 (3·07–16·62)
16:1	3·92 ± 1·05	0·45 (0·18–0·95)	4·37 ± 1·00	1·36 (0·61–3·61)
18:0	17·67 ± 5·63	1·33 (0·83–2·25)	17·85 ± 5·82	5·88 (2·52–13·60)
18:1	28·18 ± 7·57	2·61 (1·11–8·14)	41·21 ± 4·09	14·00 (5·44–28·49)
18:2	15·01 ± 6·96	1·53 (0·47–3·00)	8·54 ± 2·89	3·00 (0·81–11·03)

The above analyses showed that about 75% of pig plasma FFA was accounted for by palmitate, stearate and oleate. Mean values obtained for the rates of entry and oxidation of these individual fatty acids are shown in Table 6. Although there were insufficient observations for possible correlations between the plasma levels of the individual fatty acids and rates of entry and oxidation to be statistically examined, and in spite of the considerable variability of the results it seems likely that, on fasting, there was an increased rate of entry of palmitate, stearate and oleate and a greater contribution of these acids to total CO_2 production.

Effect of insulin

The effect of insulin on the metabolism of glucose and of palmitate was studied using the double isotope technique. The concentrations and specific radioactivities of glucose and of palmitate during the experiment are shown in Fig. 1. The sra of glucose declined more slowly than glucose concentration commencing from about 40 min from the infusion of insulin. Palmitate concentration decreased concomitantly with that of glucose accompanied by an increase in palmitate sra, indicating a depression in FFA release. When the glucose concentration fell below about 55 mg/100 ml, i.e. after about 40 min of insulin infusion, the plasma concentration of palmitate increased and its sra decreased as FFA was released into the circulation. The concentrations of both palmitate and glucose rapidly returned to their pre-infusion levels when the infusion of insulin was terminated.

Table 6. *Rates of entry of palmitate, stearate and oleate, and their contribution to total CO_2 production in fed and fasted (16 h) pigs*

Substrate	Nutritional status	Plasma level (mg/100 ml)	Total plasma FFA (mg/100 ml)	Entry rate (mg/min $kg^{0.75}$)	Approximate contribution to CO_2 (%)
[U-^{14}C]palmitate	Fed	8·0	26·1	12·0	6
		2·9	12·1	3·3	4
		1·3	4·0	1·3	1
	Fasted	14·3	58·7	12·0	24
		7·4	27·8	3·0	8
		3·9	14·9	1·0	6
[U-^{14}C]stearate	Fed	1·2	9·2	1·3	< 1
		1·9	17·2	1·3	1
		2·1	7·7	1·5	< 1
	Fasted	5·6	24·3	2·9	5
		9·9	67·1	4·7	11
		3·8	21·3	1·7	6
[U-^{14}C]oleate	Fed	1·1	5·3	1·1	3
		0·5	2·9	0·6	1
	Fasted	7·0	15·5	3·0	13
		4·5	12·1	3·4	11
		6·5	17·0	1·5	13

Cross-labelling of glucose and acetate

In four experiments in which [U-^{14}C]glucose was infused into two fed and two fasted (16 h) pigs, arterial blood samples were taken during the final stages of the infusions (after 220–240 min) and assayed for acetate. Comparison of the specific radioactivities of glucose and acetate showed that the acetate values were 12 and 15% (fed pigs) and 13 and 19% (fasted pigs) of those of glucose. In similar experiments in which the specific radioactivity of glucose was measured just before the completion of [1,2-^{14}C]acetate infusions, the values for glucose were 4 and 7% (fed pigs), and 6 and 8% (fasted pigs) of the specific radioactivity of blood acetate.

Transfer of radioactivity to lactate during labelled glucose infusions

In four experiments the sra of blood lactate at the end of the [U-^{14}C]glucose infusions (240 min) were 20 and 47% (fed pigs) and 23 and 42% (fasted pigs), of those of blood glucose.

DISCUSSION

The use of isotope dilution techniques to obtain quantitative values for the metabolism of individual substrates in whole animals is well established, but previous work on the pig has been confined to studies on the metabolism of ^{14}C-labelled glucose and acetate in the lactating sow (Gutte, Kleiber, Raggi & Black, 1961; Linzell, Mepham, Annison & West, 1969), and of ^{14}C-labelled glucose, palmitate and valine in growing pigs (Cunningham, 1967). Blood concentrations of acetate in fed and fasted pigs w

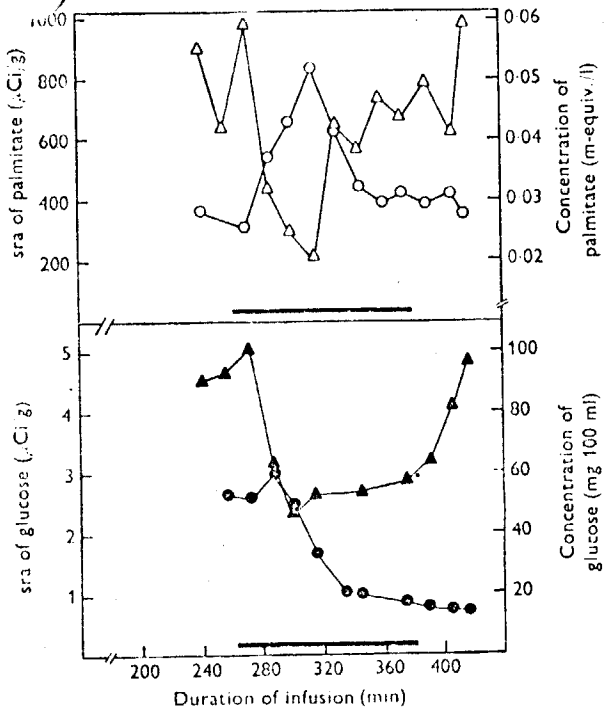

Fig. 1. Effect, in pigs, of a constant infusion of insulin on the specific radioactivity (sra) and concentrations of plasma glucose and palmitate during the continuous infusion of [U-^{14}C]glucose and [9,10-^{3}H]palmitate. ○, sra of palmitate (μCi/g); △, concentration of palmitate (m-equiv./l); ●, sra of glucose (μCi/g); ▲, concentration of glucose (mg/100 ml). The infusion rates were 0·257 μCi of [U-^{14}C]glucose and 1·420 μCi of [9,10-^{3}H]palmitate/min with a priming dose of 25 μCi of [U-^{14}C]glucose. Insulin was infused from 260 to 380 min (horizontal bar) at a rate of 2 m-units/min per kg body-weight.

not substantially different, suggesting that acetate produced from endogenous metabolism is quantitatively of more significance than acetate absorbed from the alimentary tract. Acetate is produced in the gut largely by the caecal fermentation of cellulose residues, but in the young pigs used in the present studies the level of fibre in the ration had no influence on blood acetate levels, or on acetate entry rates. The role of endogenous acetate in mammalian metabolism is obscure (see Annison, 1964), but the high acetate entry rates observed in the present studies, and the substantial contribution of acetate to total CO_2 production indicates that much of the acetyl CoA produced during metabolism must equilibrate with free acetate in blood. The equilibration of considerable acetyl CoA produced from glucose with blood acetate was shown by the transfer of radioactivity from glucose to acetate. The increased contribution of acetate to total CO_2 production in the fasted pig probably reflects the increased oxidative metabolism of fatty acids mobilized from adipose tissue.

Plasma FFA metabolism in pigs closely resembles that of other mammalian species (see Annison, 1964). Plasma levels rise during fasting (Cunningham & Friend, 1965), reflecting mobilization of adipose tissue, and the increased entry of palmitate, stearate

and oleate into body pools, and the increased contribution of these acids to oxidative metabolism in the fasted animal, are clearly shown in the present work. The plasma levels of FFA in fed and fasted pigs suggest a preferential mobilization of oleic acid on fasting, but this is not reflected in the relative increase in the entry into the plasma pool of oleate compared to palmitate and stearate. The disproportionate change in circulating levels of the medium-chain fatty acids and linoleate on fasting suggests that these acids are oxidatively metabolized to a larger extent than other fatty acids, but their quantitative significance is small relative to palmitate, stearate and oleate.

The effects of insulin on plasma FFA and glucose metabolism were studied by superimposing an insulin infusion on a double infusion of [^{14}C]glucose and [^{3}H]-palmitate. In similar experiments with sheep (West & Passey, 1967) an effect on plasma FFA metabolism could be demonstrated before blood glucose levels showed a significant decline, but in the pig blood glucose levels were almost as responsive as plasma FFA levels to insulin administration, in spite of the differences in their respective pool sizes. The initial rapid decline in plasma FFA, and the equally rapid rise in sra indicated reduced FFA mobilization, an effect eventually reversed by the sympatho-adrenal response to lowered blood glucose levels. The fall in blood glucose concentration was probably due to an increased uptake of glucose, accompanied by an increased volume of distribution (Steele, 1966). The response of the pig to glucose load and to insulin suggested that the control of glucose and fat metabolism in pigs is similar to that in other species.

The glucose entry rate determined by isotope dilution represents the sum of all the routes which contribute glucose to the total body pool, and the nutritional significance of the index lies in the relative contributions of the alimentary tract, and of other tissues. The method of measurement of entry rate assumes that the various glucose pools in the whole animal are largely in equilibrium, but clearly in the fed pig (and presumably in other non-ruminants also) a substantial fraction of the glucose absorbed from the alimentary tract may be metabolized in the liver before equilibrating with the body glucose pool, largely represented by blood glucose. That is, a substantial fraction of the total glucose pool, namely the hepatic pool, may leave the system at a lower sra than that of the total pool. The net result is that the sra of blood glucose during the infusion of labelled glucose is higher than it would be if alimentary glucose escaped uptake by the liver. In the non-ruminant substantial glucose uptake by the liver in the fed animal is well established (Shoemaker, Yanof, Turk & Wilson, 1963). The influence of hepatic glucose metabolism on the measured glucose entry rate could be calculated only if values were available for the glucose concentrations in arterial and portal blood, for the magnitude of blood flow in the portal vein and in the hepatic artery, and for glucose uptake by the liver. In the present studies we have compared glucose entry rates measured in fed pigs with the calculated rate of glucose absorption from the alimentary tract. The latter calculation assumed that dietary starch, sucrose and lactose were completely hydrolysed, and that the glucose was absorbed into portal blood. No account was taken of possible losses due to the fermentation of glucose in the alimentary tract, or of glucose metabolism in the wall of the gut. The determined glucose entry rate, however, in all cases fell short of the rate of glucose absorptio

calculated over 24 h, although entry rates were measured during the 10 h feeding period when rates of glucose absorption might have exceeded the average calculated rate. Furthermore, gluconeogenesis from protein, and from the glycerol of fat, undoubtedly makes a further contribution to total glucose production. Clearly then, the glucose entry rate seriously underestimates the amount of glucose available to the tissues of the fed animal. These results strongly suggest that glucose entry rates determined in pigs fed diets which give rise to alimentary glucose may have little quantitative significance in relation to glucose metabolism. The results for glucose oxidation based on the comparison of the sra of glucose and CO_2 in arterial blood must underestimate the extent of glucose oxidation if the hepatic oxidation of glucose represents a significant fraction of total glucose oxidation. These considerations in no way apply to the fasted animal, where there is little or no absorption of glucose from the alimentary tract. Glucose entry rates in this situation are largely the sum of glycogenolysis and of gluconeogenesis from protein, glycerol and lactate.

The use of the ratios of the sra of [^{14}C]labelled substrate and $^{14}CO_2$ in arterial blood as a measure of substrate oxidation is a gross oversimplification of a most complex situation (see Steele, Altszuler, Wall, Dunn & de Bodo, 1959; Annison *et al.* 1967). Insufficient results for the time-course of the output of $^{14}CO_2$ during the infusion of ^{14}C-labelled substrates were obtained in the present work to permit a full mathematical analysis or the calculation of a terminal asymptotic value of the specific radioactivity of $^{14}CO_2$ (Annison *et al.* 1967). The lack of the latter extrapolation, and the failure to take into account slowly equilibrating tissue pools of CO_2 leads to underestimates of substrate oxidation: the transfer of ^{14}C into other substrates which contribute to oxidative metabolism acts in the opposite direction. We must stress that the use of uncorrected $^{14}CO_2$ values provides no more than rough estimates of the contribution of substrates to total oxidative metabolism.

The values for the contribution of glucose to CO_2 production in fed (31 %) and fasted (16 %) pigs may be compared with the values of 21 and 9 % respectively obtained in the chicken (Annison, Shrimpton & West, 1969). Comparable values for the fasted dog (Steele *et al.* 1959), rat (Depocas, 1962) and man (Young, Pelligra, Shapira, Adachi & Skrettingland, 1967) were 41, 12 and 30 % respectively.

REFERENCES

Annison, E. F. (1964). In *Metabolism and Physiological Significance of Lipids* p. 289 [R. M. C. Dawson and D. N. Rhodes, editors]. London: John Wiley and Sons (Inc.).
Annison, E. F., Brown, R. E., Leng, R. A., Lindsay, D. B. & West, C. E. (1967). *Biochem. J.* **104**, 135.
Annison, E. F. & Lindsay, D. B. (1961). *Biochem. J.* **78**, 777.
Annison, E. F., Lindsay, D. B. & White, R. R. (1963). *Biochem. J.* **88**, 243.
Annison, E. F., Shrimpton, D. H. & West, C. E. (1969). In *Energy Metabolism of Farm Animals* p. 330 [K. L. Blaxter, J. Kielanowski and G. Thorbek, editors]. Newcastle: Oriel Press Ltd.
Annison, E. F. & White, R. R. (1961). *Biochem. J.* **80**, 162.
Annison, E. F. & White, R. R. (1962). *Biochem. J.* **84**, 546.
Barcroft, J., McAnally, R. A. & Phillipson, A. T. (1944). *J. exp. Biol.* **20**, 120.
Barker, J. N. & Britton, H. G. (1957). *J. Physiol., Lond.* **138**, 3 P.
Cunningham, H. M. (1967). *J. Anim. Sci.* **26**, 1332.
Cunningham, H. M. & Friend, D. W. (1965). *J. Anim. Sci.* **24**, 41.
Depocas, F. (1962). *Am. J. Physiol.* **202**, 1015.
Elsden, S. R., Hitchcock, M. W. S., Marshall, R. A. & Phillipson, A. T. (1945–6). *J. exp. Biol.* **22**, 191.

Eveleth, D. F. & Eveleth, M. W. (1935). *J. biol. Chem.* **111**, 753.
Folch, J., Lees, M. & Stanley, G. H. S. (1957). *J. biol. Chem.* **226**, 497.
Friend, D. W., Cunningham, H. M. & Nicholson, J. W. G. (1963). *Can. J. Anim. Sci.* **43**, 174.
Friend, D. W., Nicholson, J. W. G. & Cunningham, H. M. (1964). *Can. J. Anim. Sci.* **44**, 303.
Gutte, J. O., Kleiber, M., Raggi, F. R. & Black, A. L. (1961). *Z. Tierphysiol. Tierernähr. Futtermittelk.* **16**, 171.
Hanawalt, V. M., Link, R. P. & Sampson, J. (1947). *Proc. Soc. exp. Biol. Med.* **65**, 41.
Huggett, A. St G. & Nixon, D. A. (1957). *Biochem. J.* **66**, 12 P.
Jones, G. B. (1965). *Analyt. Biochem.* **12**, 249.
Laurell, S. (1957). *Acta physiol. scand.* **41**, 158.
Linzell, J. L., Mepham, T. B., Annison, E. F. & West, C. E. (1969). *Br. J. Nutr.* **23**, 319.
Sampson, J. & Graham, R. (1943). *J. Am. vet. med. Ass.* **102**, 176.
Shoemaker, W. C., Yanof, H. M., Turk, L. N. & Wilson, T. H. (1963). *Gastroenterology* **44**, 654.
Steele, R. (1966). *Ergebn. Physiol.* **57**, 91.
Steele, R., Altszuler, N., Wall, J. S., Dunn, A. & de Bodo, R. C. (1959). *Am. J. Physiol.* **196**, 221.
West, C. E. & Passey, R. F. (1967). *Biochem. J.* **102**, 58.
West, C. E. & Rowbotham, T. R. (1967). *J. Chromat.* **30**, 62.
Young, D. R., Pelligra, R., Shapira, J., Adachi, R. R. & Skrettingland, K. (1967). *J. appl. Physiol.* **23**, 734.

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PRESSOR RESPONSIVENESS FOLLOWING ACUTE ELEVATION OF SODIUM IN THE RAT[1]

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Abstract

In order to test the effects of various ions on pressor responsiveness, groups of adult rats were infused with solutions containing sufficient sodium, potassium, calcium, or magnesium to elevate the plasma concentration of these ions. The infusions were completed within 5 minutes and in no case was more than 0.5 ml. injected. The test solutions used were sodium acetate, potassium citrate, calcium chloride, and magnesium sulphate. Control solutions of ammonium acetate, ammonium sulphate, sodium chloride, and sucrose were also infused. In no case was the amount of salt infused sufficient to affect the blood pressure during the infusion. The blood pressure response to 1 γ of norepinephrine or to 20 mU. of pitressin was recorded before infusion and again at the end of the infusion. A significant effect was observed only with sodium acetate. This consisted of a profound suppression of the ordinary pressor response to both norepinephrine and pitressin. The effect was not due to the acetate ion and hence may be specifically referable to the high plasma sodium. These results support our previous conclusions that pressor responsiveness and sodium mobility are causally connected.

The studies of many workers have made it evident that sodium metabolism is in some way associated with blood pressure regulation. While most of the evidence suggests that the relation is causal in nature this inference is still far from proved.

With this problem as our central concern we have recently investigated pitressin and norepinephrine, two dissimilar pressor agents, and observed that both caused similar shifts of sodium and potassium (and water) which coincided with the rise and reversed with the fall of blood pressure (1). Inferring that these relations are causal, the theory was presented that the regulation of the blood pressure depends, *inter alia*, on the sodium transport systems broadly defined. This report presents further evidence that the relations are indeed causal.

The present experiments were designed to induce acute alterations in the ionic composition of the extracellular fluid using minimal volumes of infused solutions and then to study the response to pitressin and norepinephrine in

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the presence of such altered environments. If, as we have suggested, the ionic shifts are the determinants, not the result, of vasoconstriction, the blood pressure response might be immediately affected by the manipulation of any involved ion. This report demonstrates that it may be the acute alteration of the extra-/intra-cellular relations of the sodium ion which profoundly modifies the pressor response both to pitressin and to norepinephrine.

Methods

Adult male albino rats of an inbred Wistar strain weighing 300 g. or more were used throughout. Light ether was found to be the most satisfactory anesthesia for all operative purposes. Infusion of the test solutions was made through a polyethylene tube implanted in a femoral vein and connected to a variable speed syringe-type infusion apparatus. The apparatus could be adjusted to deliver a predetermined volume at a desired rate. Blood pressure was recorded continuously from a femoral artery connected by an implanted polyethylene tube to a Statham pressure transducer recording through a Brush amplifier and oscillograph.

The salts used to test the effects of specific ions were chosen particularly for their solubility. The aim was to inject a very small total volume (0.5 ml. or less) containing sufficient amounts of the ion to elevate the plasma concentration a significant amount within a 5 minute interval without at the same time affecting the blood pressure curve during the 5 minutes. The following salts were infused: (*a*) sodium chloride, isotonic; (*b*) sodium acetate, 4.2 *N*; (*c*) ammonium acetate, 4.2 *N*; (*d*) potassium citrate, 2 *N*; (*e*) calcium chloride, 2 *N*; (*f*) magnesium sulphate, 2 *N*; (*g*) ammonium sulphate, 2 *N*. In addition, sucrose, 2 *M*, was infused as a control for the osmotic factor.

The response to norepinephrine (Levophed bitartrate, Winthrop Stearns) was measured, using 1 γ, as the salt, injected intravenously in 0.1 ml. of saline into the femoral vein. The response to pitressin was measured similarly using 20 mU. (milliunits) in 0.1 ml. of saline prepared fresh each day from powder (Parke, Davis). In all cases the preinfusion response was compared with the postinfusion response in the same rat permitting both internal and external control of the experimental arrangement.

Plasma was analyzed for sodium or potassium by flame photometry, for calcium using a modification of Lehmann's method (3), and for magnesium using Clayton yellow (2).

Experimental

The Effect of Selected Infusions on the Plasma Concentration of Specific Ions

The final choice of solutions and concentrations was based on a large series of preliminary trials. It depended on the demonstration of a significant elevation of the specific ion within the physiological range, i.e., without affecting the continuously recording blood pressure tracing. The effect of the solutions selected on the final concentration of the specific ion is shown in Table I.

TABLE I

THE EFFECT OF FOUR SOLUTIONS ON THE PLASMA CONCENTRATION OF SPECIFIC IONS
(The volume of infusion was administered in 5 minutes)

Infusion	Vol., ml.	Plasma conc. Preinfusion	Plasma conc. Postinfusion	Rise	No. animals
Na acetate, 4.2 *N*	0.5	148 ± 2 meq./l. Na	162 ± 2	14 ± 2	8
	0.25	148 ± 2	Inconsistent change		4
K citrate, 2 *N*	0.10	4.0 ± 0.12 meq./l. K	4.9 ± 0.12	0.9 ± 0.12	8
$CaCl_2$, 2 *N*	0.12	9.2 ± 0.6 mg% Ca	17.0 ± 0.8	7.8 ± 0.8	8
$MgSO_4$, 2 *N*	0.05	1.2 ± 0.4 mg% Mg	2.4 ± 0.3	1.2 ± 0.3	8

NOTE: ± = standard error.

The Effect of Selected Infusions on the Pressor Response to Norepinephrine

Ten solutions were infused in this experimental arrangement. The results are presented in Table II. Of the 10, only sodium acetate produced a significant effect. This consisted of a profound suppression of the ordinary response to norepinephrine. The effect apparently was not due to the volume infused, nor to the osmotic effect involved, nor to the acetate moiety as judged from the results with the control infusions. Apparently it was specifically referable to the sodium ion.

TABLE II

THE EFFECT OF VARIOUS INFUSIONS ON THE BLOOD PRESSURE RESPONSE TO 1 γ NOREPINEPHRINE BITARTRATE INJECTED INTRAVENOUSLY

Infusion	Vol., ml.	Maximum pressor response, mm.Hg: Preinfusion Systolic	Preinfusion Diastolic	Postinfusion Systolic	Postinfusion Diastolic	No. animals
NaCl, isotonic	0.25	28 ± 3 (Mean)		28 ± 3 (Mean)		8
Na acetate, 4.2 *N*	0.25	24 ± 3	21 ± 3	*7 ± 2	*7 ± 2	8
Na acetate, 4.2 *N*	0.50	33 ± 9	31 ± 5	*8 ± 1	*11 ± 2	4
NH_4 acetate, 4.2 *N*	0.25	44 ± 4	34 ± 2	43 ± 4	32 ± 2	6
K citrate, 2*N*	0.10	36 ± 5	29 ± 3	44 ± 6	31 ± 4	10
$CaCl_2$, 2 *N*	0.06	33 ± 4	30 ± 3	32 ± 3	28 ± 2	8
$CaCl_2$, 2 *N*	0.12	28 ± 3	26 ± 2	27 ± 3	24 ± 3	8
$MgSO_4$, 2 *N*	0.05	31 ± 3	28 ± 2	24 ± 3	20 ± 2	8
$(NH_4)_2SO_4$, 2 *N*	0.05	36 ± 3	32 ± 3	43 ± 7	34 ± 5	6
Sucrose, 2 *M*	0.50	35 ± 7	27 ± 4	39 ± 5	29 ± 3	8

NOTE: ± = standard error. $*p < 0.01$.

The smaller volume, 0.25 ml., of sodium acetate which produced an inconsistent plasma sodium elevation was equally effective in depressing the norepinephrine response as was the larger, 0.5 ml., infusion.

The Effect of Selected Infusions on the Pressor Response to Pitressin

Seven solutions were infused in this experimental arrangement. The results are shown in Table III. Again, only sodium acetate produced a significant effect consisting of a marked suppression of the response to pitressin. As before, the effect was apparently due to the sodium ion.

TABLE III

THE EFFECT OF VARIOUS INFUSIONS ON THE BLOOD PRESSURE RESPONSE TO 20 mU. PITRESSIN INJECTED INTRAVENOUSLY

Infusion	Vol., ml.	Maximum pressor response, mm.Hg: Preinfusion Systolic	Preinfusion Diastolic	Postinfusion Systolic	Postinfusion Diastolic	No. animals
NaCl, isotonic	0.25	21 ± 4	34 ± 3	18 ± 2	31 ± 3	8
Na acetate, 4.2 *N*	0.25	17 ± 2	27 ± 3	*9 ± 1	*19 ± 1	8
Na acetate, 4.2 *N*	0.50	38 ± 6	36 ± 4	*19 ± 8	*16 ± 2	4
NH_4 acetate, 4.2 *N*	0.25	27 ± 3	29 ± 4	26 ± 4	26 ± 4	10
K citrate, 2 *N*	0.10	22 ± 2	29 ± 3	18 ± 4	29 ± 3	8
$CaCl_2$, 2 *N*	0.12	36 ± 4	39 ± 2	39 ± 6	40 ± 4	8
$MgSO_4$, 2 *N*	0.05	19 ± 2	26 ± 3	20 ± 3	25 ± 3	8

NOTE: ± = standard error. $*p < 0.01$.

Discussion

These experiments provide answers to certain problems and at the same time raise new ones. The demonstration that excess sodium modifies the pressor response to both norepinephrine and pitressin tends to support our previous conclusion that observed ionic shifts and blood pressure regulation are causally connected. They do not support the idea that the ionic shifts are the result of the vasoconstriction.

The results of the experiments are puzzling, however. The literature dealing with sodium and blood pressure has associated *chronic* excesses of this ion with hypertensive states. Here, in contrast, an *acute* excess of sodium is associated with a diminution in responsiveness to pressor agents. Two possible explanations suggest themselves: (*a*) the rapid infusion of sodium saturates the transport systems so that they cannot respond further; or (*b*) the amounts of sodium inside or outside the cell are not as important as the concentrations relative to one another. Our previous work fits with the second idea in a general way. We have noted that a fall in blood pressure coincides with an elevation in sodium outside the cell, a rise in pressure with a fall in extracellular sodium. It would be rash, however, to attempt any final explanation at this time, although our experiments provide good reason to believe that sodium transport may be intimately and causally concerned with blood pressure regulation. An elucidation of the details of the mechanisms involved is still necessary.

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References

1. FRIEDMAN, S. M., FRIEDMAN, C. L., and NAKASHIMA, M. Circ. Research (In press May, 1957).
2. HIRSCHFELDER, A. D. and SERLES, E. R. J. Biol. Chem. **104**, 635 (1934).
3. LEHMANN, J. Scand. J. Clin. & Lab. Invest. **5**, 203 (1953).

15300

Sources of Energy for Intestinal Smooth Muscle Contraction.*†

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The rhythmic contractions of a segment of rabbit small intestine, suspended in glucose-free Tyrode solution, were found by Rona and Neukirch[1,2,3] to undergo a gradual and progressive decrease in amplitude; at which time, the addition of certain substances (glucose, mannose, pyruvic, lactic, acetic, butyric, β-hydroxybutyric and oxaloacetic acids) served to increase and maintain the amplitude of contraction, the extent of the increase varying with the substance added. They attributed the progressive decrease in the height of the contractions in the absence of glucose to gradual depletion of the endogenous stores of energy-yielding substrates. The effectiveness of a substance in restoring amplitude was considered a measure of its ability to serve as a source of energy for smooth muscle contraction. More recently, Feldberg and Solandt,[4] and Feldberg,[5] in extending the work of the earlier investigators, have arrived at the same conclusions.

Except for the studies cited, the procedure introduced by Rona and Neukirch has not been utilized for the investigation of the intermediary metabolism of smooth muscle. If their assumption is correct, that the restoration of amplitude is evidence of the ability of a substance to provide energy for smooth muscle contraction, then the method would appear to be unusually well suited for such studies. The restoration of contractility would comprise a more exacting and reliable criterion for the evaluation of a substrate, than is provided by a rise in oxygen consumption alone. The latter criterion is frequently undependable; the former furnishes direct evidence that the energy provided by a substance under study is actually utilizable for smooth muscle contraction.

In the present study the method has been employed for a more extensive investigation of potential energy-yielding substrates, selected on the basis of modern concepts of intermediary metabolism, and particular attention has been given to the possible usefulness of fatty acids.

Methods. The standard preparation consisted of a fresh segment, approximately 4 cm long, from the upper portion of rabbit small intestine. The segment, with its lumen open, was suspended in a 50 cc muscle chamber, and attached to an isotonic muscle lever. The lever exerted a tension of 4 g, and recorded the contractions of the longitudinal muscle on a kymograph. The intestinal segment was bathed in glucose-free Krebs-Henseleit solution,[6] maintained at 37.5°, and aerated with 95% O_2 - 5% CO_2 to obtain a pH of 7.4.

Prior to testing each substance, the spontaneous rhythmic contractions of the longitudinal smooth muscle were allowed to decrease to about 1/10 to 1/20 of the original amplitude. This usually required less than 1 hour and introduced no change in contraction frequency. At this point, the test substance, dissolved in a small quantity of Krebs solution, was added to the muscle chamber. All acids were used in the form of sodium salts. For each substance tested, the minimal concentration was determined which brought about the maximal amplitude of contraction of which that substance was capable. This increase in amplitude was then compared on a percentage basis with the maximal change which resulted from the addition of glucose to the same intestinal preparation.

Results. The record of a typical experiment is reproduced in Fig. 1. It illustrates the gradual decrease in amplitude and average tonus which takes place in the absence of substrate as well as the restoration of both on the addition of glucose (200 mg %) when the amplitude had fallen to about 1/10 to 1/20 of the original value. The amplitude and average tonus began to increase within a minute of the addition of glucose, and the maximal amplitude was reached within 10 minutes, and maintained thereafter. In this experiment, the maximal amplitude following glucose was greater than that initially exhibited; in other experiments, the amplitude attained after the addition of glucose was either equal to, or greater than, but never less than, the original amplitude. Not discernible in the tracing because of the reduction in size, are the number of contractions per minute given by the intestinal segment and the constancy of this frequency throughout the experiment, despite marked changes in amplitude. Under our experimental conditions, the contractions averaged 17 per minute with most of the intestinal segments used. This uniformity of contraction rate throughout the experiment simplified the comparison of the relative effectiveness of different substrates in providing energy for contraction, since the only variable introduced consisted of a change in amplitude

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1 Neukirch, P., and Rona, P., *Arch. f. Physiol. (Pflüger's)*, 1912, **141**, 555.

2 Rona, P., and Neukirch, P., *Arch. f. Physiol. (Pflüger's)*, 1912, **146**, 371.

3 Rona, P., and Neukirch, P., *Arch. f. Physiol. (Pflüger's)*, 1912, **148**, 273.

4 Feldberg, W., and Solandt, O. M., *J. Physiol.*, 1942, **101**, 137.

5 Feldberg, W., *J. Physiol.*, 1943, **102**, 108.

6 Krebs, H. A., and Henseleit, K., *Z. f. Physiol. Chem.*, 1932, **210**, 33.

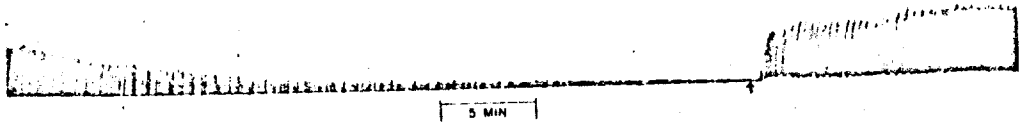

Fig. 1.
Kymograph tracing showing decrease in amplitude of intestinal contractions in glucose-free solution, and restoration of contraction amplitude on addition of glucose (at arrow) to provide a concentration of 200 mg %.

The second step in the experimental procedure—that is, the comparison of the relative effectiveness of different substrates in supporting smooth muscle contractions—is illustrated in Fig. 2. The results obtained with 4 substrates are expressed in semi-diagrammatic fashion, each contraction wave representing approximately 17 contraction waves in the actual kymograph tracing. With the amplitude after addition of glucose as the standard of reference, the relative effectiveness of the other substrates is as follows: pyruvic acid, 100%; butyric acid, 50%; and enanthic acid, 20%.

In Tables I and II, are assembled the results obtained with a number of substances which proved capable of restoring, in varying degrees, the amplitude of smooth muscle contraction. In Table I, the height of the response is compared with that produced by glucose, and, for each substance, represents the maximal response which it can elicit. Table II lists the minimal concentration of each substrate required to bring about its individual maximal increase in amplitude.

Table III lists those substances which failed

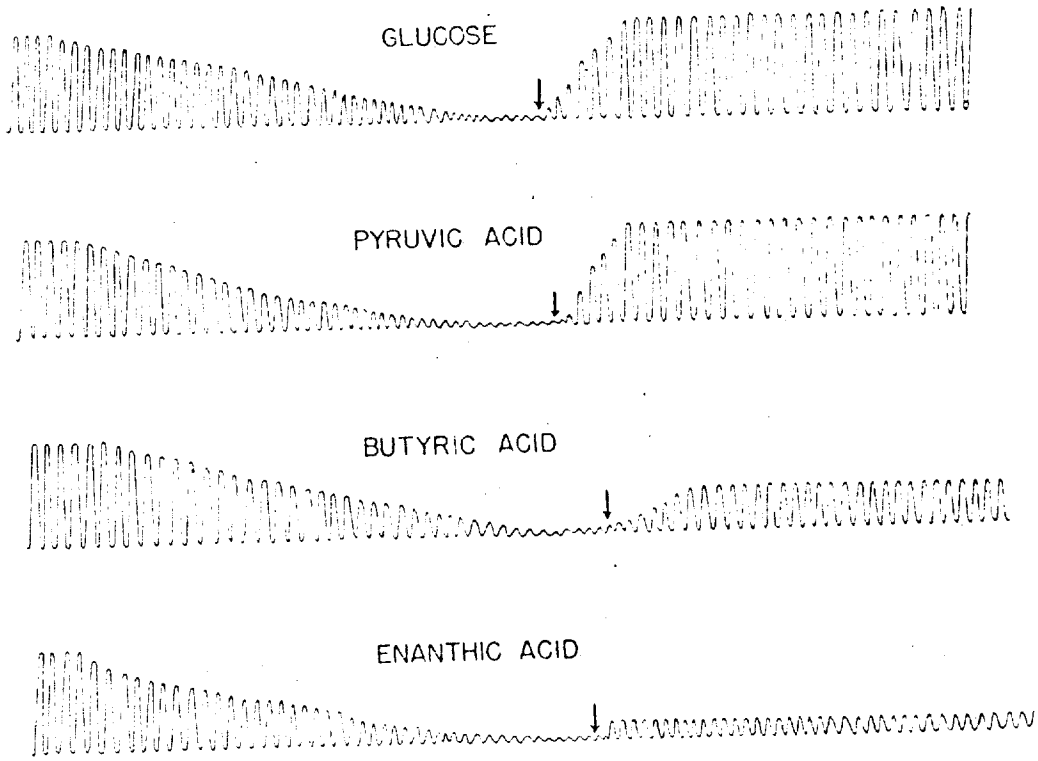

Fig. 2.
Semi-diagrammatic representation, based on kymograph tracings, illustrating relative effectiveness of glucose, pyruvic, butyric, and enanthic acids in restoring amplitude of contraction of intestinal smooth muscle after prior exposure to a glucose-free solution.

TABLE I.
Substances which Increase Amplitude of Contraction.

Substance	Response compared with that of glucose
	%
Glucose	100
Mannose	70-90
Acetic acid	100
Pyruvic acid	100-110
Oxaloacetic acid	75-100
d,l-Lactic acid	20-40
Acetoacetic acid	20-30
Butyric acid	40-60
Caproic acid	40-60
Caprylic acid	40-60
Pelargonic acid	35-45
Enanthic acid	15-20
Valeric acid	5-10

to increase the amplitude of contraction. Each was tested over a range of concentration extending from 10 to 100 mg %, and found to be without influence on the gradual reduction in tone and amplitude of contraction characteristic of the preparation in the absence of substrate. There were 2 exceptions: acetaldehyde and glyceraldehyde, in concentrations of 100 mg %, exerted a marked inhibitory effect, as evidenced by the rapid and virtually complete cessation of all contractile activity. The inhibition induced by acetaldehyde could not be overcome by the subsequent addition of glucose, pyruvate, acetate or lactate, whereas that caused by glyceraldehyde could be overcome by pyruvate, acetate, or any of the fatty acids listed in Table I, but not by glucose or lactate. In the case of citric acid, which removes Ca ions from solution to form a weakly dissociable

TABLE II.
Minimal Concentrations Necessary for Maximal Increases in Amplitude of Contraction.

Substance	Minimal concentration in mg %
Glucose	100-125
Mannose	125-150
d,l-Lactic acid	20-30
Oxaloacetic acid	20-30
Acetic acid	10-15
Pyruvic acid	10-15
Butyric acid	4-5
Valeric acid	3-4
Caproic acid	2-3
Enanthic acid	1-2
Caprylic acid	0.5-0.75
Pelargonic acid	0.5

TABLE III.
Substances which Fail to Increase Amplitude of Contraction.

Galactose	Propionic acid
Fructose	Hydroxyacetic acid
Glycogen	Citric acid
Glucose-1-phosphate	Succinic acid
β-Phosphoglyceric acid	Fumaric acid
Glycerol	α-Ketoglutaric acid
d,l-Glyceraldehyde	Glycine
Acetaldehyde	l(+)-Alanine
Ethanol	l(−)-Aspartic acid
Acetone	l(+)-Glutamic acid

(Ca citrate)⁻ complex, the precaution was taken to add sufficient Ca ions (as $CaCl_2$) to replace those removed, in order to avoid changes in muscle tonus which would otherwise have resulted from a deficiency of Ca ions in the medium.

Fig. 3 is a graphic presentation of the maximal increases in amplitude observed with the series of fatty acids from acetic through pelargonic. The greatest increase, which equalled that given by glucose, was obtained with acetic acid. The remaining even-carbon fatty acids (butyric, caproic and caprylic) all produced an identical increase in amplitude, which was approximately 50% of that of acetic acid. On the other hand, the effects of the odd-carbon series of fatty acids were strikingly different. Propionic acid was devoid of effect. The changes in amplitude induced by the other members of the odd-carbon series were less (valeric, enanthic and pelargonic) than those obtained with even-carbon fatty acids, and differed in extent with each compound. The magnitude of response increased as the chain-length increased. Thus, the approximate increase with valeric acid was 10%, with enanthic acid 20%, and with pelargonic acid 40% of that observed with acetic acid.

Experiments of a preliminary character have been carried out to investigate the mechanisms responsible for the differences exhibited by odd- and even-carbon fatty acids. If β-oxidation takes place in smooth muscle, as our experiments suggest, the smaller fragments resulting from this type of degradation of the even-carbon fatty acids would all be utilizable for muscle contraction; whereas the odd-carbon fatty acids would leave a non-utilizable propionic acid residue. Since the

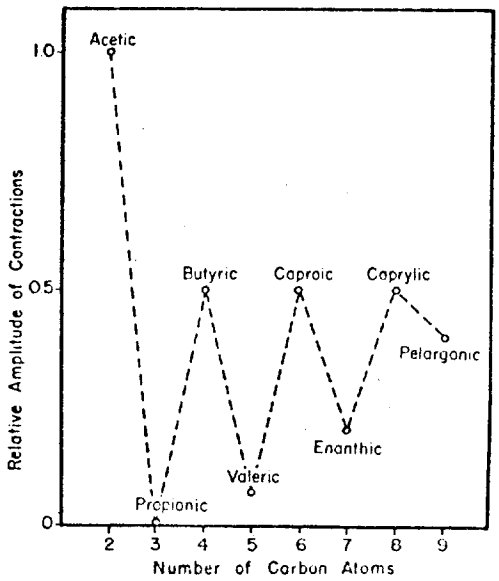

Fig. 3.
Relative effectiveness of odd- and even-carbon fatty acids in restoring amplitude of contraction of intestinal smooth muscle following prior exposure to glucose-free solution.

propionic residue would be formed at the surface of the postulated β-oxidation enzyme, it might conceivably compete for this surface with the unaltered fatty acid or its oxidizable fragments. This concept was explored by adding propionic acid in concentrations of 10-30 mg % to muscle preparations contracting under the influence of butyric or caprylic acid, in concentrations of 5.0 and 0.75 mg % respectively. In each instance, 30 mg % of propionic acid served to reduce the amplitude of contraction by about 80%. This inhibition, however, could subsequently be overcome by doubling the concentrations of butyric or caprylic acid.

The observation was also made that relatively high concentrations of enanthic, caprylic and pelargonic acids completely inhibited smooth muscle contraction. The effective concentrations varied with different intestinal preparations and were as follows: enanthic 50-200 mg %; caprylic 25-150 mg %; and pelargonic 20 mg % (1 experiment). This inhibition was not overcome by the addition of glucose, pyruvic acid, or acetic acid. However, it proved to be a reversible inhibition, since contractility was restored when the fatty acid was washed out and replaced with fresh Krebs solution.

Discussion. 1. *Mode of Action of Substances Which Restore Contractility.* Those substances which restore contraction amplitude may act either as substrates or pharmacological agents. All the evidence so far accumulated, although indirect, appears to favor the substrate concept. Rona and Neukirch[1] observed a reduction in glucose and mannose content during a 2½-hour period of active contractions. More specific evidence against a pharmacological action was provided by Feldberg and Solandt.[4] The addition of acetylcholine during a period of diminished activity in a glucose-free Tyrode solution produced a relatively small increase in tonus, sustained for less than a minute. However, following the restoration of amplitude by glucose or pyruvate, acetylcholine produced a marked and well-sustained increase in tonus. From this it was inferred that glucose and pyruvate do not increase smooth muscle activity by increasing the production of acetylcholine, and that this agent could exert its pharmacological effect only when energy for contraction was provided by appropriate substrates. These experiments have been confirmed and extended in our laboratory, with the observation that large and sustained increases in tonus may also be obtained with acetylcholine after the restoration of amplitude by fatty acids, such as acetate and butyrate. Although this evidence is indirect, we favor the substrate concept over the pharmacological, particularly in view of the conformity of much of the data obtained with prevailing views of intermediary metabolism. The discussion of our results has therefore been based on the concept that the capacity of a substance to augment and sustain contraction amplitude is derived from its ability to provide energy for smooth muscle contraction.

2. *Carbohydrate Series.* Of the hexoses studied, only glucose and mannose yielded energy for contraction; galactose and fructose were ineffective. In 2 respects our findings differ from those of Feldberg and Solandt.[4] We failed to find the increase in reactivity to acetylcholine reported by these investiga-

tors to follow the addition of fructose to a glucose-free medium. We also found mannose to be 70-90% as effective as glucose, in contrast with the 25-30% effectiveness which they observed. This latter discrepancy may be due to the fact that they did not use maximal increases in amplitude as the basis for comparison.

The excellent substrate effect of pyruvic acid is in accord with the Meyerhof cycle. The smaller effect produced by lactate is of interest. The lack of effectiveness of glycogen, glucose-1-phosphate, and β-phosphoglyceric acid need not be considered evidence against the occurrence of the Meyerhof cycle in smooth muscle since it is questionable whether the muscle cells are permeable to these substances. The same reservation holds for the ineffectiveness of glucose-6-phosphate and fructose-1,6-diphosphate, reported by Feldberg.[5]

3. *Acetic Acid.* This proved to be an excellent substrate, producing contraction amplitudes equal to glucose and pyruvate. This is compatible with recent observations on the utilization of acetate by a variety of tissues. The pathway for the oxidation of acetic acid in smooth muscle remains obscure; it does not appear to be oxidized to hydroxyacetic acid, since the latter had no substrate effect.

4. *The Krebs Tricarboxylic Acid Cycle.* Of the acids postulated in this cycle the following were tested and found ineffective: citric, α-ketoglutaric, succinic and fumaric. Oxaloacetic acid exerted a good substrate effect. However, the exceptional behaviour of oxaloacetic acid prompts the suggestion that it may have exerted its effect only after decarboxylation to the readily oxidizable pyruvic acid. The ineffectiveness of succinic acid is in accord with *in vitro* metabolic experiments with strips of dog intestinal smooth muscle (unpublished data). In these experiments carried out at reduced O_2 tensions, the increased O_2 uptake in the presence of succinic acid failed to increase the concentrations of phosphocreatine and adenylpyrophosphate, the high energy phosphates essential for muscle contraction. These results provide no support for the participation of the postulated tricarboxylic acid cycle in the activity metabolism of smooth muscle, and, by the same token, would exclude the cycle as a pathway for the oxidation of pyruvic and acetic acids.

5. *Amino Acids.* In conformity with the earlier observations of Neukirch and Rona,[2] the amino acids studied were found to exert no substrate activity. This was of particular interest with respect to alanine and aspartic acid, since their corresponding keto-acids, pyruvic and oxaloacetic, are extremely effective. Apparently, intestinal smooth muscle is unable to deaminate these amino acids to their keto forms.

6. *Ketone Bodies.* Neukirch and Rona[2] had previously reported a small increase in amplitude with β-hydroxybutyric acid. We have observed an effect of similar magnitude with acetoacetic acid, but none with acetone. Should the lack of utilization of acetone prove to be a general phenomenon, it might provide an explanation for the readiness with which acetone appears in the urine even with mild disturbances in fat metabolism.

7. *Fatty Acids.* The difference in the effects observed with odd- and even-carbon fatty acids strongly suggests that β-oxidation takes place in intestinal smooth muscle. Thus, butyric, caproic and caprylic, which can all be completely degraded to 2-carbon fragments, are all equally effective as substrates. On the other hand, propionic acid, which is not susceptible of β-oxidation, is ineffective; and the remaining odd-carbon fatty acids studied, all of which would leave a propionic acid residue on β-oxidation, have a smaller effect than those of the even-carbon series.

An explanation for the identical effects of the even-carbon fatty acids may be that they all undergo β-oxidation at the same enzyme surface; and that, once this surface is saturated with any one of them, β-oxidation then proceeds at the same rate. Support for this hypothesis is provided by the fact that once a maximal increase in amplitude is induced by any one of the series, the subsequent addition of another results in no further increase.

Our experimental results also permit a tentative explanation for the smaller effects of the odd-carbon acids (valeric, enanthic, pelargonic) as well as for the increase in

amplitude with the increase in chain-length. The β-oxidation of odd-carbon fatty acids should in every instance leave a non-utilizable propionic acid residue. Propionic acid has been found to reduce the effectiveness of even-carbon fatty acids as substrates, an inhibition which can be overcome by raising the concentration of the utilizable fatty acid. This suggests the competition of propionic acid for the surface of the β-oxidation enzyme. With the odd-carbon series, the competition between the propionic residue and the unaltered acid and its β-oxidizable fragments, would explain the smaller effects of this series. This competition should become less effective as the chain-length increased, since the β-oxidizable portion would progressively increase relative to the propionic acid residue.

One other factor may also contribute in part to the greater effectiveness of the odd-carbon fatty acids of longer chain-length. It was observed, for the whole series of fatty acids, that, as the chain-length increased, progressively smaller concentrations were required to produce maximal amplitude effects. An explanation for this finding may reside in the greater affinity of the longer-chain fatty acids for the β-oxidation enzyme surface, a concept which derives support from the increase in the adsorption coefficients of fatty acids at surfaces, as chain-lengths increase. With respect to the odd-carbon series, such an affinity relationship would act to the advantage of the longer-chain members in their competition with propionic residues.

No information is at present available as to the usefulness of higher fatty acids, such as oleic, palmitic, and stearic, as substrates for smooth muscle contractility, because of the extremely low solubility of their sodium salts in Krebs solution. They were ineffective in dilute suspensions.‡

Summary. A number of substances have been studied with respect to their influence on the contraction of excised segments of rabbit small intestine. The results have been discussed in relation to present concepts of intermediary metabolism. Particular attention has been given to fatty acids, from propionic through pelargonic. With the exception of propionic, all appear to provide energy for smooth muscle contraction; and the characteristics of their behaviour were compatible with the concept of β-oxidation.

‡ Since this manuscript was submitted, oleic acid has been found to partially restore contraction amplitude. It was introduced as a sodium oleate sol (100 mg %) in water along with enough NaCl to maintain isotonicity. The effective concentration of oleic acid could not be determined because of its partial precipitation.

We wish to acknowledge the technical assistance of Mathilda Fischl, Alice Kantor, and Rita Baffa.

Furia, T.E. (Ed.) 1972
Handbook of Food Additives
Chemical Rubber Company, Cleveland,Ohio
p.147

Int. Congr. Occupational Health, 14th, Madrid
2: 723-7
(1964)

ASPIRATION HAZARDS OF PETROLEUM PRODUCTS

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We became interested in the aspiration hazards of petroleum products when it came to our attention in reviewing poisoning reports among children that kerosine and other petroleum solvents and furniture polish containing mineral seal oil stood second only to aspirin as a cause of accidental poisoning in children. Our interest was to know more about the cause of these poisonings, and determine what could be done to prevent or treat them. Analysis of the reported cases disclosed that serious lung damage usually developed in these children, but if they survived the first week or so, complete recovery without sequelae was the rule. Generally these children drank kerosine carelessly left by their parents in accessible places in water glasses or 'coke' bottles. Furniture polish was gulped from an open furniture polish container left on a low table while mother was occupied elsewhere – often times only for a few brief moments on the telephone. By far the majority of the cases occurred in children under 5, often 2 or under.

A review of the literature indicated that there were two schools of thought on this problem. The first believed that the kerosine or furniture polish entered the lung directly by aspiration at the time of swallowing, or by subsequent vomiting. The other believed they were absorbed into the blood stream in a sufficiently high concentration to cause injury to the pulmonary capillaries from the high blood concentration.

Our approach to this problem, developed by one of us (HWG), was a direct experimental one. First, kerosine was administered to experimental animals by stomach tube in high doses (10 cc/kg). Figure 1 shows two rats which received 40 doses of 5 cc each of kerosine by stomach tube. Except for some excoriations around the anus, these animals are quite healthy. Examination of their lungs showed perfectly normal lungs, as in the center lungs of Figure 2. Contrast this, however, with the lungs on either side removed from rats which died following the instillation of 0.1 cc of kerosine directly into the trachea. Here, hepatization of the lungs has occurred, with acute cor pulmonale having developed. Figure 3 contrasts the microscopic picture of normal lung tissue with the bloody exudation into the lungs, following direct instillation of small amounts (0.1-0.2 cc) of kerosine directly into the trachea.

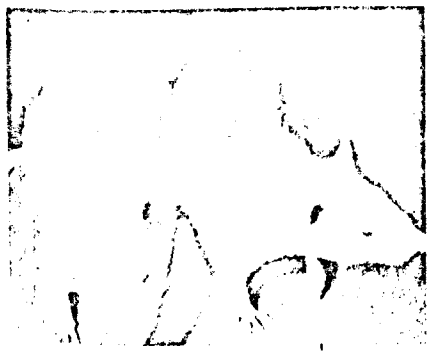

Fig. 1. Two rats which received 40 doses of 5 ml each of kerosine by stomach tube.

Fig. 2. Gross appearance of rat heart and lungs after aspiration of 0.2 ml of kerosine (right and left; center normal).

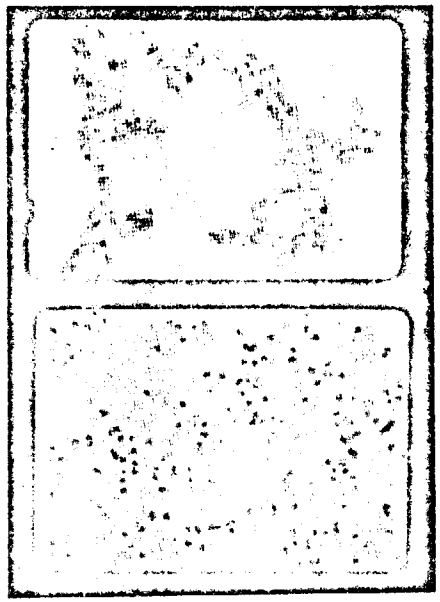

Fig. 3. Microscopic appearance of rat lungs after aspiration of 0.2 ml kerosine (upper one normal). Magnification ×430.

Figure 4 shows a rabbit dosed with so much kerosine by stomach tube that his fur actually became wet with kerosine excreted from the anus. Yet this rabbit was otherwise healthy. A fraction of this dose placed directly in the trachea resulted in death of the rabbit with extensive lung damage. Similar experiments have been performed in chickens, with comparable results. Figure 5 shows extensive damage in the medial dependent portions of chicken lungs from kerosine placed directly in the trachea.

We believe the lessons learned from these simple, direct experiments are so self-evident that they need only emphasis. Petroleum products which enter the stomach and remain there are essentially nontoxic. Thus, the *oral* LD_{50} of kerosine (Figure 6) is 28,350 milligrams/kg. On the other hand, if the kerosine is aspirated into the lungs, either due to gagging at the time of ingestion, or by subsequent vomiting, the LD_{50} (Figure 7) is between 400 and 600 milligrams/kg. It thus seems self-evident to us that kerosine in the stomach should be left there, because the hazard of leaving it there is nil, but the hazard of aspiration during attempts to remove it from the stomach is so great as to seriously endanger the patient. If aspiration into the lungs has already occurred, the only thing to be accomplished by inducing vomiting or passing a stomach tube is the possibility of aspirating more kerosine into the lungs with further lung damage. Since this has been established experimentally in all species of animals tested, there is no reason to doubt that the same principles apply in the human. Supportive therapy, with oxygen and antibiotics, should be given if aspiration has already occurred.

Extending this work further, Dr. Gerarde has developed a standardized test for the aspiration hazard of a petroleum hydrocarbon. A rat weighing between 200 and 300 grams is anesthetized with ether. Its jaws are held open with forceps (Figure 8) and the tongue pulled forward. 0.2 ml of the test material is placed at the back of the pharynx and the nares held shut during inspiration. This causes aspiration of the material into the lung. Mortality at 24 hours is measured and found (Figure 9) to be a function of viscosity. If the viscosity exceeds 90 or 100 SSU at 100°F, the material produces no deaths at 24 hours. On the other hand, if the viscosity is less than this, aspiration with subsequent deaths does occur. If the lungs are removed and weighed 24 hours after dosing in this fashion, it is found that lung weights are increased for those materials which produce deaths (Figure 10). If the lung weight (normal for the rat 1–1.5 gms) does not exceed 3 grams, deaths do not occur. Using this technique, mixtures of kerosine and lubricating oil were found to present no hazard if the viscosity exceeded 58 SSU at 100°F. Thus no mortality (Figure 11) or increased lung weight (Figure 12) occurred when the viscosity exceeded 58. We believe that this clearly demonstrates that the aspiration hazard of a petroleum hydrocarbon is related to its viscosity, and can be simply tested by this technique.

Finally, a modification of this technique was developed to test the aspiration hazard of petroleum hydrocarbon aerosols. Figure 13 shows kerosine being sprayed directly into an anesthetized rat's mouth. As shown in Figure 14, the actual spraying is done with the nozzle close to or actually in the animal's mouth. As much as 1 cc of kerosine (5 times the amount which causes death and lung damage when instilled as a liquid directly in the trachea) was without mortality or increase in lung weight when aspirated as an aerosol (Figure 15). We thus conclude that petroleum hydrocarbon aerosols are without hazard from aspiration.

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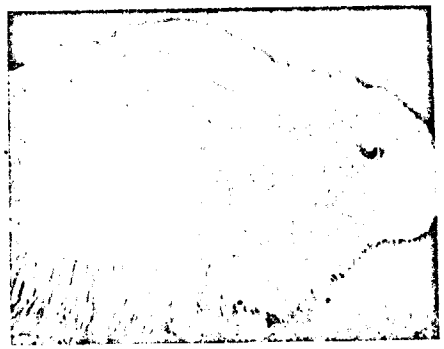

Fig. 4. Rabbit dosed with large doses of kerosine. Fur wet with kerosine excreted through anus but rabbit otherwise healthy.

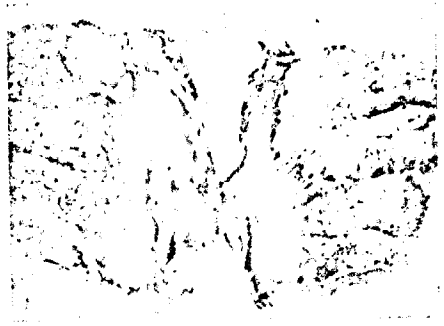

Fig. 5. Chicken lungs after aspiration of kerosine. Note hemorrhagic areas in medial and dependent regions.

ORAL TOXICITY OF COMMON LIQUID CHEMICALS		
CHEMICAL	LD-50 (mg/kg)	TOXICITY CLASS
ACETIC ACID (RAT)	3,310	SLIGHTLY TOXIC
LACTIC ACID (RAT)	3,730	SLIGHTLY TOXIC
ISOPROPYL ALCOHOL (RUBBING ALCOHOL)(RAT)	5,840	PRAC. NON-TOXIC
ACETONE (RAT)	9,750	PRAC. NON-TOXIC
ETHYL ALCOHOL (RAT)	13,660	PRAC. NON-TOXIC
GLYCERINE (RABBIT)	27,000	REL. HARMLESS
KEROSENE (RABBIT)	28,350	REL. HARMLESS

Fig. 6. Oral toxicity of some common materials, including kerosine.

MALE ALBINO RATS
INCREASING DOSES OF KEROSINE (ASPIRATION)

Dose (ml)	Mortality (Hours After Dosing)						
	1	2	6	24	48	72	16 DAYS
0.05	0/10	0/10	0/10	0/10	0/10	0/10	*1/10
0.10	0/10	1/10	3/10	4/10	4/10	4/10	4/10
0.15	0/10	2/10	6/10	7/10	8/10	9/10	9/10
0.20	0/10	3/10	7/10	9/10	9/10	9/10	9/10
0.25	4/10	7/10	10/10	10/10	—	—	—

*DIED ON 10th DAY

Fig. 7. Mortality of rats (numerator) over number of rats treated (denominator) with increasing doses of kerosine.

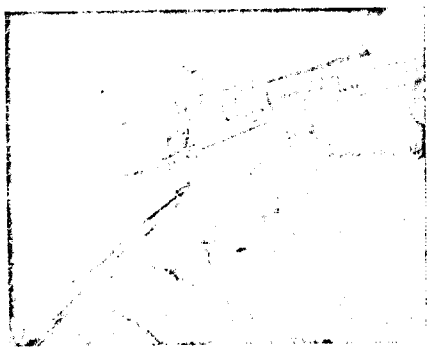

Fig. 8. Method of administering liquids for testing aspiration hazard in the rat.

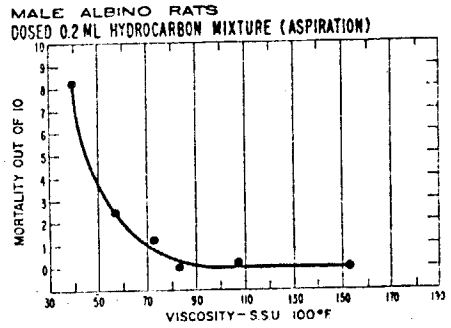

Fig. 9. Relationship of mortality of rats after aspiration to viscosity of the hydrocarbon aspirated.

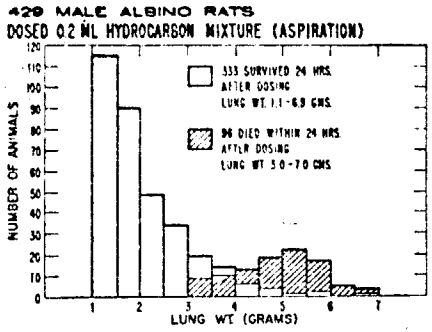

Fig. 10. Relationship of lung weight in rats 24 hours after aspiration to survival. Deaths occur only if the lung weights exceed 3 gm.

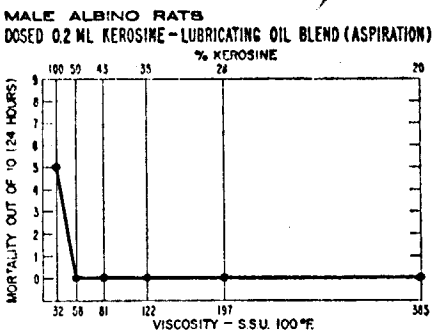

Fig. 11. Effect of increasing the viscosity of kerosine by adding lubricating oil on the survival of rats which aspirated the mixture. Note that 50% of kerosine with a viscosity of 58 SSU at 100°F caused no deaths.

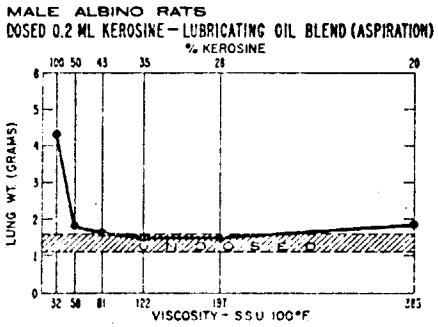

Fig. 12. Relationship of lung weight to viscosity of aspirated kerosine-lubricating oil mixture. Note that lung weights are not significantly increased if viscosity exceeds 58 SSU at 100°F.

Fig. 13. Method of administering aerosol.

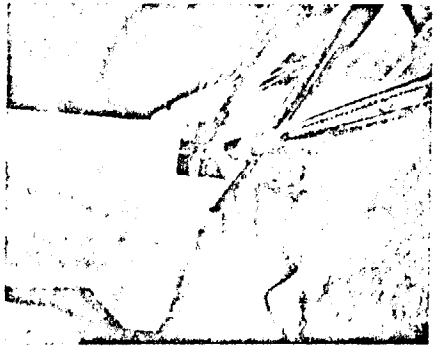

Fig. 14. Same as Figure 13, but in practice the aerosol orifice is placed close to rat's mouth.

MALE ALBINO RATS
ASPIRATED 1.0 ML OF KEROSINE AEROSOL

(Sacrificed 24 Hours After Dosing)				
Mortality	Clinical Observations After Dosing	Lung Weights (Grams)	Avg.	Gross Pathology
0/10	NO EVIDENCE OF SYSTEMIC INTOXICATION OR PULMONARY DISTRESS LOCAL IRRITATION AROUND EYES & NOSE CLEARED RAPIDLY	1.18, 1.27, 1.22, 1.06, 1.43, 1.28, 1.47, 1.16, 1.43, 1.30	1.28	* LUNGS NORMAL ON INSPECTION

LUNG WEIGHTS UNDOSED: RANGE 1.1–1.5 GRAMS AVG. 1.3

Fig. 15. No mortality or increased lung weight in rats following aspiration of an aerosol of 1.0 ml of kerosine. Contrast this with Figures 2 and 7.

Conclusions

Light petroleum hydrocarbons, such as kerosine and certain solvents, or mineral seal oil, have a very low order of toxicity if ingested and kept in the stomach. However, if aspirated directly into the lungs they cause extensive lung damage and death. Therefore, the induction of vomiting or pumping out the stomach is contraindicated following petroleum hydrocarbon ingestion unless extreme precautions, such as the insertion of an intratracheal tube, are taken to avoid aspiration. Heavier petroleum hydrocarbons are without aspiration hazard (if the viscosity exceeds 60–100 SSU at 100°F). A simple test for determining the aspiration hazard using mortality and lung weights at 24 hours is described. Tests of petroleum hydrocarbon aerosols show them to be without aspiration hazard. Kerosine or other petroleum solvents should not be left in open, unmarked containers, such as water glasses or 'coke' bottles where they can be accidentally ingested and aspirated by small children. Furniture polish likewise should not be left opened when small children are around.

Resumen, Abstract, Résumé, Vol. IV, No. 196

severe injury of erythrocytes in view of the high affinity of acetic acid towards their lipoid coating. The erythrocytes shrink, grow pale and are finally completely destroyed.

In the blood vessels, extensive thrombosis is noted, and a brown coloring of the blood coming in direct contact with the acid. The liquid blood takes on a salmon-pink color and contains hemoglobin which can be detected by spectroscopy.

The strong erythrolysis leads, through a hematinemia, to a true hemolytic (anhepatic) icterus ("hematin icterus", not "bilirubin icterus"). In rapid sequence, changes take place in the spleen by hemoglobin imbibition, in kidneys by hemoglobin casts and hemoglobin infarction with epithelium necroses, and hemoglobinuria sets in. The action of the acid leads to the removal of alkali from the blood and thus to a general acidity; caustic burns of the gastrointestinal tract cause large blood losses; blood viscosity changes cause circulation troubles and difficulties in heart activity; bleeding time is prolonged.

As a result of the action of acetic acid vapors or acid breathing, injuries of the respiratory tract are almost always noted, even when changes in the digestive tract are very slight. Hemorrhagic pneumonias occur in more than half of the fatal poisoning cases. In animals, the inhalation of evaporating acetic acid already has a fatal effect.

Thus, a great variety of disturbances can finally lead to death. Our case of a fatal poisoning can be included without any difficulty among such findings. The reason why this case can be particularly emphasized is the presence of peculiar changes in the liver and spleen, in the form of limited and infarct-like anemic necroses accompanied by extended vascular thromboses,

whereby beginning repair and cirrhosis-like reactions were taking place in the liver. The results of animal tests with acetic acid to induce liver cirrhosis are very contradictory; our case assumes special importance as a kind of human experiment, which could be recorded accurately from a clinical and pathological-anatomic viewpoint.

The case deals with a 41-year-old bartender, who had returned a year ago, as a result of a leg injury from a Russian prisoner of war camp in very poor condition and on crutches. He had been bed-ridden for 7 weeks due to abdominal typhus, and in spite of an initial good recovery, he slowly underwent an increasing exhaustion of strength (hyposthenia) due to alcohol and nicotine abuse. The day after he was released, the patient drank 200 cc of a 60% acetic acid solution with the intent of suicide. Severe nausea with pains in the upper abdomen and bloody vomiting set in immediately; 6 hours later, blood was found in the stool and urine. The attending physician suspected at first a bleeding gastric ulcer, and the patient was admitted to a hospital, in view of increasing pains, only 30 hours later.

Upon admission, small wounds covered with scabs were noted at the corner of the mouth and on the rear of the tongue. A burning thirst gradually appeared, along with agonizing pain. Repeated attempts to introduce a feeding probe (stomach feeding tube) always stopped when the probe reached a depth of 20-25 cm. Circulation required continuous support. After 2 days, nausea had diminished, but the burning thirst had increased in spite of grape sugar and salt infusions. The temperature was above 38°C, the pulse remained at a high level of 100-130. The blood sedimentation reaction was 9/25. Traces of protein were found in the urine, and a few erythrocytes, along with a large number of leucocytes, were found in the

sediment; urobilin, uribilinogen and bilirubin were normal.

After 5 days, the patient was transferred to the Charite laryngological clinic; the very strongly impaired circulation was now the predominant feature of the patient's sickness. The skin was definitely pale, the patient could not talk and was very short of breath; his condition was similar to one in coma. The pulse was thready and lay around 160, the temperature reached almost 40°C. The entire mucous membrane of the mouth, palate and velum, and also of the nose, was extremely dry and coated with hard crusts. An attempt to make the patient swallow on his own succeeded with considerable difficulty. The next day, crusts were removed from the mouth and nasal cavities, and these were painted with a glycerol-boric acid mixture. A feeding tube could now be applied with no difficulty. However, cardiac and circulation support, such as infusions, was only temporarily successful, and the patient died 8 days after poisoning.

Autopsy findings. Strong atrophy of fatty tissues, and clearly expressed rigor mortis. Strikingly pale, but not icteric skin, yellowish sclerae still present. Extensive grey-black crust formation around the mouth and nose; gums, oral mucosa and tongue coated with dirty-grey to brownish scabs. The throat (pharynx) strongly red-colored; larynx and epiglottis have a moderate edematous swelling. Esophagus clearly distended (enlarged); its mucosa is spotty to streaky, coated with dull grey to red-brown scabs, especially in its lower part; in some spots, the mucosa is absent altogether. The stomach is greatly contracted; the serosa is slate-grey to black with a dull shine, the walls are swollen, rigid and dry. The small stomach content is a thick red-brown, tar-like liquid with a characteristic aromatic acid odor. The mucosa is wide and folded in a

roll-shaped manner, with an irregular brown to dark-brown-red color; in the pylorus section it is extensively necrotic, brittle, blood-soaked and can be broadly detached from its substratum; in the cardia, the mucosa is better preserved, unevenly nodulated, and has a grey-brown color. The strongly contracted pylorus is sharply delineated from the duodenum; the mucosa of the latter exhibits only surprisingly small, spotty hemorrhages.

The greater omentum has a dirty yellow-brown color, and its fatty tissue shows extensive atrophy. Veins of the stomach wall and of adjacent parts of the omentum and mesentery are strikingly wide, stand out greatly, and are engorged with red-brown to blackish coagulated and finely grooved blood clusters. The lower vena cava and the lower sections of the splenic vein contain liquid, salmon-pink blood.

Finely grooved, dark red-brown and occasionally whitish spotted emboli are found in the upper part of the splenic vein and all the way up in the finer branches (rami), which in part adhere and in part are firmly attached to the wall and completely block the lumen. The spleen is slightly enlarged, with a map-like surface and cross-section structure, partially covered with yellow-grey and dark blue-red spots; the pulp is moderately loosened and can be stripped off.

The liver is slightly enlarged, with a smooth surface; through the capsules of the left and right lobes, isolated, circumscribed and irregularly blotched (spotty) parenchyma portions, up to 5-mark coin in size, can be seen, which glisten in a bright-yellow to pale brownish color; in liver sections, these parenchyma portions extend in a wedge-shaped and infarct-like manner towards the hilus, and stand out clearly from the remaining more reddish, dull glistening parenchyma, rich in blood and juice, due to their

dull grey clay-like color and strongly blurred lobular pattern. The corresponding small branches of the portal vein are occluded by dark brownish soft thrombi, which occasionally can be observed inside medium-large branches of the vein; the main duct of the portal vein contains only liquid blood. Neither here, nor in the veins, arteries and biliary tract can any pathological changes be detected with the naked eye.

Both kidneys are moderately swollen, moist glistening and rich in blood; the cortex is pale brownish and slightly turbid, and the pyramids are dark red with slight radial, brown-black streaks.

The mucosa of the bladder exhibits a dirty grey defect in the center of a limited, brightly red area.

The serosa of the colon and small intestine has a spotty violet-grey color. The colon is very wide, and the wall of the upper colon sections exhibits an edematous swelling and has a spotty dark red color; its mucosa is extensively defective and is coated with grey-red scabs. The mucosa of the lower ileum has an intense red color, and the Peyer's patches are swollen and imbibed with grey-red streaks.

The trachea and bronchi contain large amounts of viscous, brownish-red mucus; their mucosa is somewhat swollen and has a uniform, strong red color. Both lower lobes of the lungs are large and heavy and are permeated (infiltrated) with irregular, occasionally flat, red-grey and fine-grained pneumonic foci. Pulmonary pleura covers both lower lobes, especially the right one, and is dull and covered with fresh, fibrinous layers.

Autopsy diagnosis (36/47): Corrosive burns of the stomach caused by acetic acid poisoning. High-degree, hemorrhagic necrotising gastritis. Moderate scabbing with superficial ulceration and dilatation of the esophagus.

Thrombosis of the vena lienalis with infarct-like necrosis of the spleen caused thereby; thrombosis of portal vein branches with liver necroses; thromboses of omentum, stomach and mesentery veins. Extensive hemorrhagic enteritis; high-grade colitis with scab formation; pharyngitis; strong tracheobronchitis. Spotty croupous pneumonia of both lower pulmonary lobes, confluent in the right lobe; bilateral dry pleuritis; hemorrhagic edema of other pulmonary lobes. Limited necrosis of the bladder mucosa. Cyanosis and turbidity of kidneys. Dilatation of the right heart area; brown degeneration of the myocardium. Slight scar formation of aortic roots; fibrosis testis. Chronic tonsillitis. Edema of meninges; hemorrhages in the leptomeninx. Height (of patient): 176 cm, weight: 51 kg, liver 1990 g, spleen 210 g.

Microscopic findings. 1. Liver Cross-section of one of the infarct-like liver sectors shows the presence of unsharply limited, spotty-clouded parenchyma necroses in foci grouped like on a map, located on the periphery of thrombotic occluded, smaller portal vein branches. These necrotic areas are separated from the liver surface by a border of preserved parenchyma of varying width (Fig. 1). At the point where these necrotic areas come close, in a tongue-like fashion, to the liver capsule, the latter is retracted in a scarred fashion and contains a larger number of nuclei.

The emboli of portal vein branches completely occlude the vascular lumen and show a mutual stratification of erythrocytes, fibrin with thrombocytes and cellular elements, which allows us to conclude that they were formed gradually after a previous stasis. In individual spots, the vascular intima has a loose structure and is stripped of endothelium; here, the organization of a thrombus is started by connective tissue cellular elements.

Fig. 1. Partial view picture. Spotty necroses of liver tissue (a) with partially preserved tissue structure and widening of the interstice (b). Small scarred retractions of the liver capsule (c). Strong dilation of of adjacent intralobular capillaries (d). Central veins (e). Hematoxylin-eosin staining. Magnifying glass enlargement.

Arteries and liver veins are completely free of any pathological changes; in some spots, the coarser bile ducts contain in their outer wall layers small, focus-shaped round cell infiltrations.

The cloudy tissue necroses, mixed with portions of preserved parenchyma, consist of denucleated liver cell plagues, forming a mosaic-like arrangement of tightly packed beveled cubes (Fig. 2); the cellular membranes of these plagues are often greatly damaged, and their protoplasm is pale eosinophile, has a blurred granular structure, hardly transparent and permeated with isolated, nonsudanophile vesicular vacuoles. Many plagues exhibit a varying degree of diffuse, bilious impregnation, and some plagues show a finely granular, yellow-brownish disintegration pigment. The lobular structure is represented only by the quite well preserved reticular fiber framework,

which surrounds the individual cell plagues as a fine network, that can be stained deep blue by Azan; this reticular framework appears to be larger in size (increased) and coarser than the one observed in healthy liver lobules, and is particularly clearly visible around bloodless central veins, which appear to lie isolated in the tissue with their thickened and loosened (porous) vascular walls (Fig. 1).

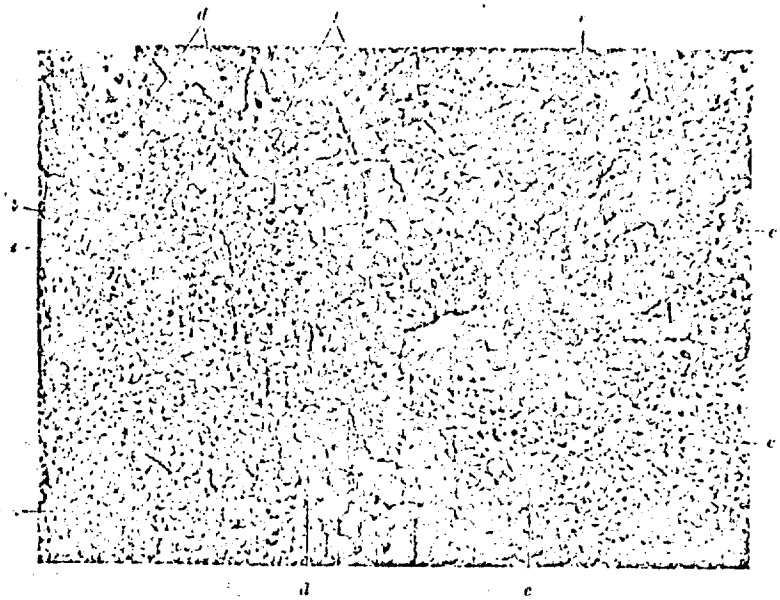

Fig. 2. Border area between necrosis and preserved liver parenchyma. Proliferation of Kupffer's cells in the area of necroses(a). Irregular, primarily peripheral destruction of adjacent liver lobules. Resorptive and proliferative inflammation in the area of the boundary sheath with proliferation of the mesenchyma(b) and the bile ducts(c), and strong round cell infiltration in front of the dissociated branches of liver cell trabeculae(d). Atrophy of the preserved liver cells(e) with dilation of capillaries; multiplication (increase) of nucleated blood cells within the capillaries and central veins(f); strong reticulo-endothelial reaction. Hematoxylin-eosin staining. Magnified 320-fold.

A capillary arrangement can no longer be recognized; only here and there, small, isolated and sparingly blood-filled capillaries can be seen in the destroyed parenchyma. Most capillaries have collapsed, and thus give the false impression of an increase (multiplication) of the lattice

framework. On the other hand, the capillary endothelia are clearly visible everywhere, and in some spots even appear to be increased and become rare only in those areas where a strong blurring of the liver cell boundaries indicates a progressive necrosis. Here and there, the capillary endothelia surround (envelop) with long, wing-like processes small fragments of necrotic liver epithelium; others have an elongated, spindle-like shape like fibrocytes and have started, by intra-acinal means, to build a tender, collagenous fibril network, without the presence of any connection with periportal connective tissue. The cell body of these capillary endothelia is mostly clearly swollen and fully packed with fine, yellowish and only partially iron-containing pigment granules. Sometimes, the swelling of the nucleus is even more clearly visible than that of the cell body. As a result of a rounding of nuclear poles, the nucleus assumes more of an elongated oval shape and exhibits fine, more marginal chromatin structures. Thus, these cells are only very little reminescent of endothelia and can hardly be differentiated from fibroblasts.

Necrosis of the liver cells is not connected with the lobular structure, and has affected (attacked) the liver lobules in a variable and irregular extent. Many lobules are affected in sectors of varying width, others in a tangential direction, while others are smoothly halved in front and behind the central vein; only isolated trabecular parts of some lobules are found in some cases (Fig. 2 and 3). Single necroses around central veins or preserved liver cells, arranged in a ring around Glisson's fields, cannot be detected anywhere; all destructions show a broad connection with the lobular periphery. The starting point of tissue damage must therefore be sought in the periportal fields.

The transition between necrotic and preserved liver epithelium is relatively sharp only at certain points. A layer, often containing a single row, of collapsed and necrotic liver cells with large-drop fatty deposits in the protoplasm, with an opaque granulation and vacuolic degeneration, and also pycnotic nuclei, separates the two layers and is permeated with bands of tightly packed, strongly pigment-loaded and fibroblast-like cells (Fig. 3).

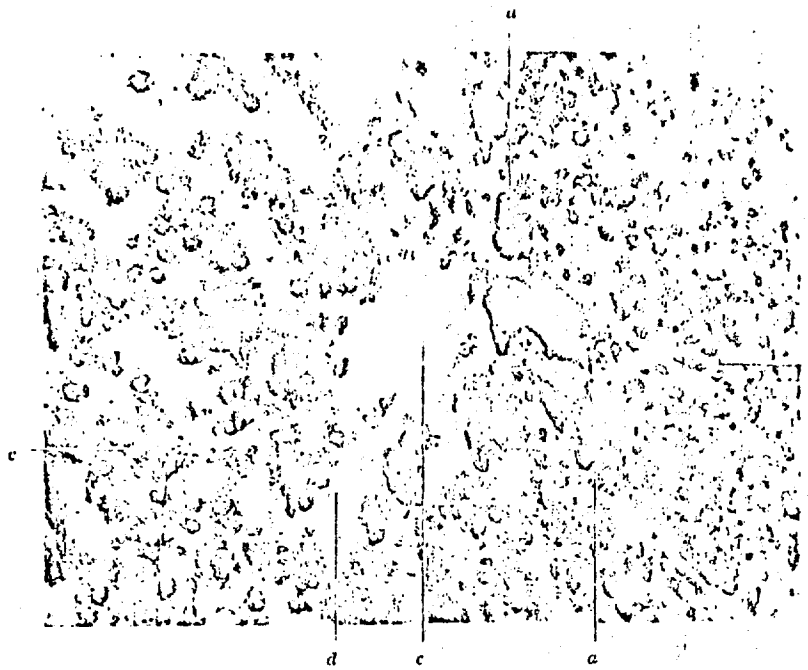

Fig. 3. Central area of a liver lobule, with partial parenchyma destruction, cutting the lobule into two halves close in front of the central vein. Atrophy, necrobiosis and large-drop fatty infiltration of liver cells in the boundary area(a). Strong and irregular proliferation of capillary endothelia in the area of destroyed parenchyma cells(b). Dissociation and small-drop fatty infiltration of preserved liver cell trabeculae(c), with dilation of intralobular capillaries (d) and the central vein(e). Sudan staining, magnified 400 times.

Mostly, however, the preserved liver parenchyma gradually terminates in the necroses (Fig. 2). The liver cell trabeculae become increasingly narrow, and the cytoplasm, at first strongly eosinophile, fades; the

nuclei become smaller and their chromatin network becomes more compact. The pericellular reticular fiber network becomes more clearly visible, splits up and dissolves, by losing its trabecular structure, into an increasingly fine-meshed network, in which liver cell nuclei, as if suspended, are still visible; these nuclei have a sparse, pale grey plasma body, which often contains vacuoles and has an elongated cuspidal shape (Fig. 4). In the mesh of the network, the intralobular capillaries, which have widened in accordance with the narrowing of cell trabeculae, have expanded in a lake-like fashion and have become filled with erythrocytes. At the same time, lacerations of the capillary wall with extravasations have occurred at some spots. Accordingly, the strongly proliferated capillary endothelia contain large amounts of iron-containing pigment, and this is sometimes the only way in which they can be differentiated from the atrophic liver epithelium.

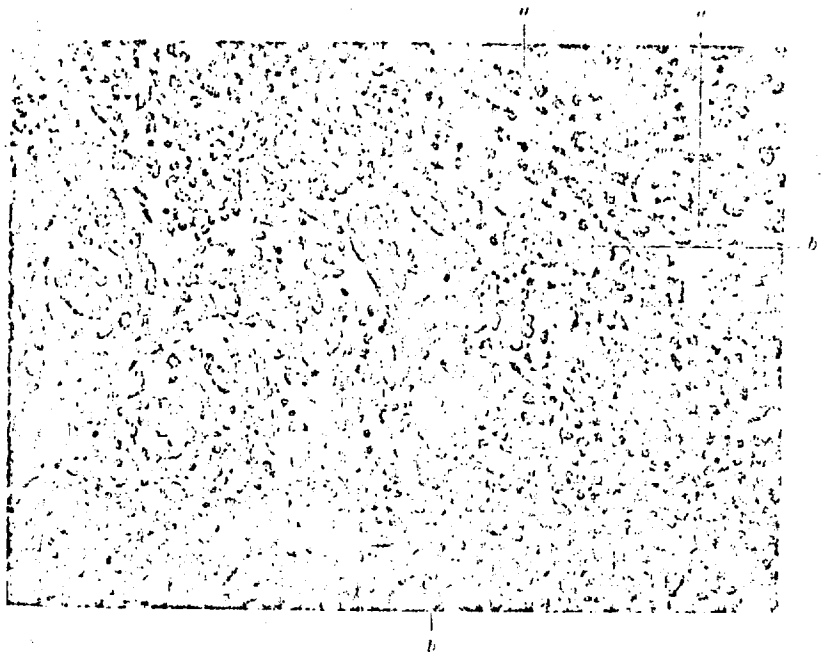

Fig. 4. Strong proliferation of the mesenchyma, slightly infiltrated with round cells, with compact structure of the fiber network. Bile duct proliferations. Collapse of liver cell trabeculae(a) with reticular proliferation of capillary endothelia and reticular fiber network(b). Hematoxylin-eosin staining. Magnified 400-fold.

However, not all marginal liver epithelia are destroyed, and many clearly show an attempt to undergo regeneration. Thus, constricted groups of liver cells or isolated, dispersed and rounded liver epithelia, with a greatly distended plasma body (of uniform finely granular structure, deeply eosinophil-visualized and having produced a bilious pigment), are found in front of some times plump, club-shaped distended and strongly twisted processes of liver cell trabeculae (Fig. 5). These liver cells have sharply defined cell boundaries, the nuclei are clear and always enlarged, sometimes swollen to giant size and then inflated and containing vacuoles. Frequently, several nuclei are also found in a common plasma body as a sign of amitotic cell division. Mitotic figures, however, are infrequent.

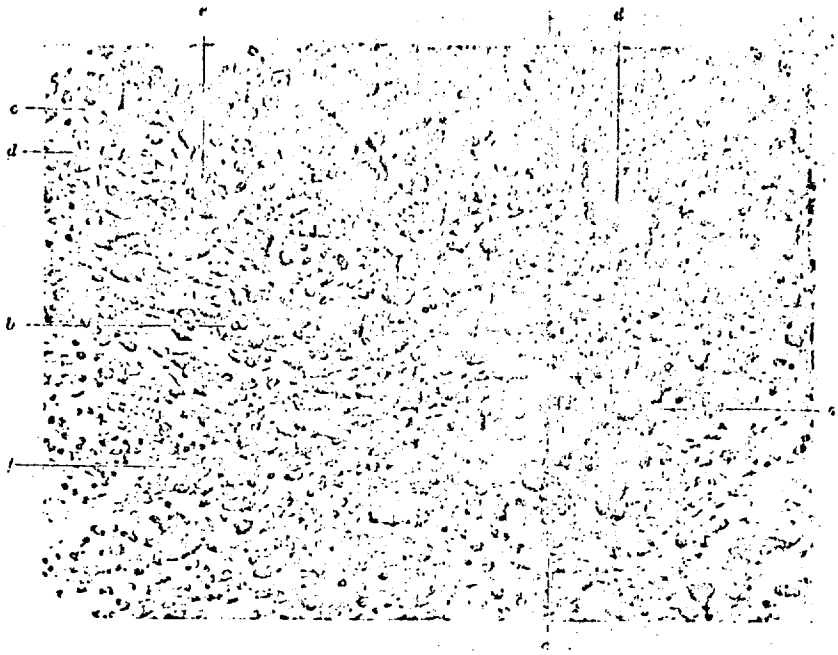

Fig. 5. Widened periportal field from the proliferation zone area with proliferations of the mesenchyma cells, phagocytosis of iron-containing pigment(a) and numerous new sproutings of capillaries(b). Increase of fibrous connective tissue. Atrophy(c) and regeneration products(d) of adjacent liver cells, including also ring-shaped proliferations(e). Bile duct proliferations(f). Staining with Berlin blue and nuclear fast red. Magnified 400-fold.

The intralobular capillaries of liver lobules adjacent to necroses are strongly dilated even a long way in the healthy stage; they contain surprisingly few erythrocytes and these frequently exhibit anicytosis and anisochromia, but they are filled with large amounts of cloudy, finely granulated protein bodies, often in the form of cylinder-like spouts. Dispersed in between are numerous nucleated cell elements, which in part can be considered as blood cells, mostly leucocytes; a large portion of these elements consists of proliferated, rounded and desquamated capillary endothelia in the sense of an endothelium proliferation (Fig. 2). The capillary walls are loosened (porous) and in some spots separated (or elevated), and their fibrils have an unsharp profile. In the pericapillary area, isolated lymphocyte-like or fibroblast-similar cellular elements are found, as well as veil-like plasma streaks.

The periportal fields are greatly widened (expanded) and are connected (linked) with tender endothelium proliferations, located between and in front of the collapsed lobular processes, to a connective tissue organization zone, which has a varying width and which envelops the necroses in a mantle-like fashion. This zone consists mostly of fibroblast-like cells, and to a lesser extent of histiocyte cells, fully loaded with mostly iron-containing pigment; this zone shows a dense mixture with polymorphonuclear leucocytes, lymphocytes and particularly with large numbers of plasma cells. Between the cells lies a compact (dense) network of fine-fibrillar connective tissue, which is in a continuous communication with the reticular fiber network of both preserved and necrotic liver portions, and which can be stained deep blue with Azan (Fig. 4). This network corresponds only in part to the collapsed and split reticular fiber networks of liver lobules,

and represents to a large extent a young, collagenous fibrous tissue. A dense permeation with numerous, mostly newly formed capillaries completes the picture of this network as that of a typical granulation tissue.

Under the pressure of this newly formed tissue, individual adjacent liver lobules exhibit a narrowing and parallel stratification of liver cell trabeculae. In combination with the often marginal position of central veins in partially damaged liver lobules and with the increase of intralobular connective tissue mentioned above, the formation of pseudolobuli can already take place.

The terminal ramifications of the portal vein in Glisson's fields are greatly expanded (widened) in a lake-like fashion, and are tightly filled primarily with round cells, mostly polymorphonuclear leucocytes and also a few fibroblast-like cells.

The bile ducts exhibit extensive proliferations in the area of peri-portal fields. These proliferations are mostly in the form of small, longitudinal or transverse epithelial tubes with no lumen, arranged chain-like in double rows, with a tendency to arrange themselves around a circular or oval lumen. In doing so, these tubes often surround nuclear fragments, polymorphonuclear leucocytes and also granular pigment. In many places, these tubes surround, in the form of small elongated cell groups, the processes of liver cell trabeculae or even penetrate between the bulb-like hypertrophied trabecula tips.

After from necroses, the liver parenchyma exhibits a definite narrowing (constriction) of the liver cell trabeculae; the central veins and adjacent capillaries are always somewhat wide and largely filled with erythrocytes. Centrally located liver cells contain a large amount of brown lipochrome

and also small amounts of fine to coarse fat droplets. In the periportal fields, clearly visible proliferations and strong round cell infiltrations can also be detected here. The small branches of the portal vein are wide and filled with large numbers of nucleated blood corpuscles, while the branches of liver arteries exhibit small, focal, adventitial infiltrates. The biliary ducts exhibit extensive proliferations.

2. Hilus lienalis. Amidst regular (normal) fat tissue, several larger veins are transversely affected, and their lumen is completely filled with fresh, dark-red-brown blood clots (thrombuses). The intima is intact all around: lymphocytic elements lie either scattered or lumped together, in the adventitial tissue. Nothing special is noted in the arteries.

3. Kidney. The glomeruli are normal. The epithelia of the coiled renal tubules exhibit a large degree of alterations, such as turbid swelling, and contain in some spots fine-droplet fatty infiltrations (deposits); their nuclei are often necrobiotic. Epithelial damage is considerably smaller in the loops, intercalaries and collecting ducts. Isolated epithelia of the main parts, as well as desquamated and denucleated epithelia, sometimes contain red-brownish, granular plasma inclusions. The lumen of loops, intercalaries and especially of collecting ducts contains pads (spheres) of a coarse, light-brown granula, frequently mixed with dead epithelium and then shaped like cylinder casts (Fig. 6); these can be stained a dark brownish color, according to Lepehne, similarly to blood, and light-green with cresyl violet. These renal tubules, due to the expansion and flattening of their epithelia, can sometimes be confused with blood-filled vessels. The adjacent epithelia proper also often carry fine, granular, black-brownish protoplasma inclusions. In the area of medullary papillae, a deep-blue

granula can be detected in the interstitium with Berlin blue in isolated cases. Individual loops also contain isolated granulocytes. The vascular system exhibits a strong blood congestion.

Fig. 6. Cross-section through the long Henle's loops of a renal papilla. Cylinder-like casts and dilatations of renal tubules by hemoglobin (Hemoglobinuria). Edema of interstitium. Lepehne. Magnified 320-fold.

The transfer (passage) of acetic acid into the circulation system takes place mainly in the stomach. A spasm of the pylorus prevents the further penetration of acetic acid and causes a prolonged stay in the stomach. The strength of the caustic (corrosive) action on the gastric wall, and therefore the intensity of poisoning, is directly related to the amount and concentration of the solution supplied, and also to the momentary filling state of the stomach. Mitschling visualized a complete impregnation of the gastric wall with diffusion of acetic acid into the free abdominal cavity; in this way, acetic acid also penetrates vascular walls and is mixed with circulating blood. Erythrocytes agglutinate and thus cause circulatory troubles. The additional hemolysis taking place causes, by means of liberated

products promoting coagulation, extensive thromboses in vessels and capillaries, as well as a general hemoglobinemia. Heine injected highly concentrated acetic acid into the femoral vein of dogs and achieved extensive dark thromboses stretching from the injection site to the right ventricle. The obstruction of stomach vessels accelerates its destruction process. Esau observed, after applying acetic acid compresses, necroses of the skin, as a result of capillary thromboses, also at the tips of healthy fingers.

The thrombosis of vessels occurs too slowly over a stasis to be able to bar successfully the circulation system against the effects of acetic acid. Peripheral blood is flooded very soon with free hemoglobin. Already 6 hours after poisoning, "bloody" urine (hemoglobinuria) is observed. We must certainly reckon with the presence of acetic acid in peripheral blood, as can be assumed from the peculiar odor of organs noted by many authors. This fact is supported in our case by the type and freshness of lung involvement, which forces us to assume the presence of hematogenous causes, along with aspiration of acid vapors. According to Vincenzo, acetic acid is eliminated, among other things, through the bronchial mucosa. On the other hand, no specific mention is made anywhere of hemorrhagic pneumonias in hemoglobinemias due to other causes. Boix observed in rabbits, after peroral administration, the crystallization of acetic acid clusters in liver veins. A rapid breakdown of acetic acid in the blood appears improbable, since it occurs as a normal decomposition product in blood and urine, and since it is hard to oxidize and undergoes combustion with relative difficulty.

Vascular thromboses are found in all venous outflow circuits of the stomach, as well as in their neighboring opening (junction) areas, the upper splenic vein (V. gastroepiploica sin. and Vv. gastricae breves) and

the lower portal vein (V. gastroepiploica dextra and V. coronaria ventriculi).

The selection of vascular areas in the liver affected by thrombosis, and thus the blotched appearance of the necroses, depends upon the specific flow directions of the separately running influx blood columns within the portal vein flow, and also upon the fact that the liver always performs its function in a mosaic-like fashion.

Cardiovascular disorders of the liver alone exert no after-effects on the parenchyma. In the case of truncal obstruction of the portal vein, the arteria hepatica takes over the blood supply to a sufficient extent. A continuous (prolonged) blockage (occlusion) of the portal vein acts in the sense of a disuse atrophy, as was confirmed by the experiments of Reddingius, and causes an irregular connective tissue formation and sometimes considerable sclerosis, but rather in the form of atrophy than cirrhosis.

Schulz and Muller observed, after portal vein thrombosis, a uniform atrophy of the liver with interstitial induration, but without any pathological changes of the liver cells. The fibrillar tissue was very rich in cells and contained, in addition to large numbers of spindle-shaped nuclei and round cells, numerous bile duct proliferations. On the other hand, necroses of the parenchyma were noted by Heller, Joannovicz, Kobrich and Verse. Gruber (II), Buday (II), Loeb (II), Brie and Josselin de Jong observed necroses with a "light cirrhosis". But neither Joannovicz nor Kretz were able to produce a proliferation in the periportal connective tissue, let alone the production of cirrhotic changes.

Infarct necroses of the liver are formed only when the finest ramifications of the portal vein are also blocked beyond on the other side of interlobular arteriovenous anastomoses, in "terminal" portal vein thromboses,

since this also causes a blockage of arterial influx.

The purely mechanical nature of these necroses was confirmed by Raestrup, who injected into the portal vein of guinea pigs corpuscular toxic products enclosed in capsules which dissolved only after a long period of time (delayed action capsules), so that these products could not exert their action immediately; he achieved in this manner an obstruction of numerous vessels by thrombotic encasing of the injected foreign bodies and, as a result, extensive liver necroses. Ribbert observed necroses after injection into the portal vein of substances blocking blood vessels, such as bone oil and agar-agar solution. Schmorl describes multiple necroses as the consequence of a primary toxic thrombosis of the smallest ramifications in the portal vein during puerperal eclampsia. It is certain that necroses caused by immune sera are also partially due to the formation of hyaline thrombuses.

Liver necroses as a result of a toxic injury of the parenchyma have been frequently observed, and have been experimentally produced in animals by injection into the portal or mesenteric vein of a large variety of highly concentrated poisons such as ether, chloroform, carbon tetrachloride, alcohol, phosphorus or other products.

All products having a severe damaging effect on blood were found to be special liver toxins. According to Jaffe, all blood toxins must also be considered as liver toxins, in view of their damaging effect on erythrocytes. Thus, severe degenerations of the parenchyma were achieved by Jaffe with hemoglobin solutions, and by Joannovicz, Pearce and Wooldridge with hemolytic immune sera. Fahr assumes that eclampsia also exerts a toxic parenchyma injury.

Reports on liver changes after acetic acid administration to humans are extremely scarce. In most cases, only an acute hyperemia of the liver was noted. The only case, which perhaps corresponds to the one described here, was reported by Gerhartz senior (?), although unfortunately without any microscopic findings; he observed cyanotic infarcts of the liver on the 3rd day after peroral acetic acid poisoning. A classical atrophic liver cirrhosis, confirmed by autopsy results, was noted by V. Bergmann in a man who many years ago, in order to avoid military service, had been drinking for weeks every day in the morning 1/2 liter of vinegar on an empty stomach, and who faithfully stated that he never drank any alcohol. "Vinegar drinking" had been readily used earlier in weight reduction treatments, and resulted mostly in chronic digestive troubles, stomach pains with colic, diarrhea, anemia and shortness of breath (chronic acetism).

Animal tests for the production of liver injuries by acetic acid have been carried out by various authors, and these tests were prompted by the efforts of Boix to check the toxic action on the liver of isolated intestinal contents modified by chronic dyspepsia. Upon feeding butyric, acetic, lactic and other acids to rabbits, acetic acid was found to be particularly toxic and irritating and by far the most dangerous of all acids; it caused, within 36 days, a strong degeneration of the liver epithelia, clearly visible round cell infiltrations of periportal spaces, and an extensive blotched or striated sclerosis all the way up to an atrophic liver cirrhosis, comparable to the one described by Laennec. On the other hand, alcohol was found to be toxic, but not irritating to the liver tissue; however, it had a strong irritating effect on the gastric mucosa. Studies of the stomach content of alcohol drinkers and dyspeptic patients, as well as of the vomit after poor

digestion, revealed the presence, in considerable amounts, of admixtures of acetic acid and similar acids. Wine, a frequent cause of liver cirrhosis, also contains acetic acid among other products. Boix subjects the term "alcoholic cirrhosis" to severe criticism, and would like to supplement it by the term "dyspeptic" cirrhosis; in this case, he thinks about a possible enterogenous conversion of alcohol, non-irritating to the liver, to liver-irritating organic acids, thus, among others, about a rearrangement of alcohol to acetic acid inside the stomach.

v. Bergmann also assumes that secondary products formed in the intermediate degradation of alcohol may be the cause of liver changes due to alcohol, and also considers acetic acid in this case.

The results obtained by Boix have been extensively debated and have not always withstood post-examinations. Josselin de Jong was unable to produce with acetic acid those broad features of interacinic tissue which were described by Boix; he obtained merely a scant cirrhosis formation. Joannovicz obtained, by administration of acetic acid through a sound (stomach) tube, very light infiltrations of the periportal connective tissue and an atrophy of liver cells, however, with no signs of more severe degenerations. Vincenzo noted changes in the liver vessels by using small acid doses, administered over a long period of time; Koelsch obtained only a fatty degeneration of the liver after poisoning with organic acetates. Attempts made by v. Bergmann to induce liver injuries in animals with acetic acid were completely unsuccessful.

Boix and Joannovicz stress in their experiments the strong degenerative action of acetic acid on liver cells. Since we are dealing here with feeding tests, an indirect action over a hemolysis or intrahepatic thromboses

cannot be excluded. Proof for a direct action, damaging liver cells, was therefore provided for the first time by Parisot and Harter, who were able to produce focal liver cell necroses in the common bile duct (choledocus) by injection of dilute acid solutions; their rabbits died at different intervals of 1-18 days and exhibited, along with extensive and well defined parenchyma necroses (clearly visible already after 24 hours), a strong hyperemia of the veins and intralobular capillaries, large round cell infiltrations of periportal fields, especially around biliary ducts and small veins, changes in the bile duct epithelium, and a definite fresh, sclerosing connective tissue in the periportal spaces with radiation into intralobular clefts similar to a definite liver cirrhosis. The spleen was greatly enlarged, blood-congested and pigment-loaded, and its follicles showed the presence of central necroses.

Experiments with acetic acid were carried out almost exclusively on rabbits, mostly by feeding, and the results were not always in agreement. These results are subject to reservations when applied to man, since great difficulties are experienced due to the profound differences in the intermediate metabolism of man and animals. An important path, namely injections into the portal vein, as has been done so often with other toxic products, has unfortunately not been followed. Here, our observation can perhaps be used to supplement previous experience, since, in our case, a massive flooding of acetic acid into the liver through the portal vein can be definitely assumed. We recognize quite clearly that our observation only represents a single, selected point in the course of what happens during the action of acetic acid on the liver, although at a point in time when particularly interesting changes are to be expected, in the form of a

degeneration coming to an end and a beginning regeneration. In general, the beginning of a regenerative reaction of the liver after toxic injury is assumed to take place around the first to third day, and to reach its peak around the 6th to 14th day. We are able to confirm in part the results obtained by Parisot and Harter by means of injections into the choledochus. When we summarize our findings, we get the following facts. The action of acetic acid on liver tissue occurs in stages, including 3 steps:

1. The primary effect is a direct damage of the parenchyma.
2. The secondary effect is a prolonged, damaging action by thrombosis.
3. An injury also of the mesenchymal sections of the liver, and thereby stimulation of connective tissue proliferation and scar formation.

As signs of tissue damage, we find blotched (spotty) and irregular necroses in limited and infarct-like areas (Cameron's "toxic infarct"), as well as extensive degenerative and dystrophic changes, primarily in liver epithelia. At the same time, individual liver lobules are affected, starting from the periphery and often in sharply restricted sectors, in the form of non-regenerating pseudolobuli, so that a rapid course of injury with a narrow spatial limitation of the injurious product must be assumed; it is not conceivable that such profound and sharply limited injuries, dissecting the lobules, are caused only by thromboses of portal vein branches, since, from the nature of thromboses, their gradual formation through a stasis must be assumed. We would much rather attribute to the thromboses of portal vein ramifications, in the first stage, the role of a lagging marking of the path which acetic acid must have followed while flooding the liver. In accordance with the degree of damage inflicted to the tissue, we can detect all the transition points between the complete homogenous-cloddy disinte-

gration of liver epithelia and atrophy of liver cell trabeculae, with the collapse of lobules or lobular sections and the vicarious dilation of intralobular capillaries. In addition, a direct damage of capillary walls, with a primary ectasis and secondary pressure atrophy of liver cell trabeculae, also certainly plays a role. Fatty infiltrations of adjacent liver cells are not only a consequence of the toxic action, but must also be considered as the result of changes in fat metabolism based on extensive disorders.

As signs of a regeneration, we find numerous, primarily amitotic cell divisions of liver epithelia with the development of giant cells and small nodular hyperplasias, an increase (multiplication) of reticular fibers in the sense of an inter- and intralobular collapse fibrosis, and also very numerous bile duct proliferations. According to observations made by Manwaring and Clara, the proliferation of bile ducts and of interlobular connective tissue takes place at the same time as the proliferative processes in the parenchyma. Herxheimer and Tholldte pointed out that the bile duct proliferations always run parallel to those of the periportal connective tissue, whereas, on the contrary, "pseudotubuli" are found primarily during acute atrophy of the liver. We also find more "pseudotubuli" than typical bile ducts, but we are unable to draw a sharp separation line between these two elements. Rather, all transition forms between typical bile ducts and "pseudotubuli" can be demonstrated, and are found here in particularly beautiful form and in large numbers. The change in shape (form) of "pseudotubuli" discloses a certain regularity, namely that, with increasing distance from typical bile ducts, the epithelia assume a more plane cubic to spindle shape, the nuclei become more oval and clear, the lumen has a

smaller and more irregular shape, often lying in an eccentric position or even disappearing completely. If we note at the same time that the "pseudotubuli" lie in a particularly dense compact manner in front of the peripheral processes of liver cell trabeculae, and sometimes even edge themselves in between these processes, it becomes apparent how strongly the bile ducts strive to reestablish a connection with still functioning or newborn liver cells. The abundance of "pseudotubuli" proliferations must in this case be evaluated as an excess production.

However, it appears unlikely that proliferations originate only from interlobular bile ducts; rather, we can assume that interlobular and perilobular (septal) bile ducts, as well as their intermediate parts, proliferate at the same time to the biliary capillaries, whereby the intermediate parts constitute the major portion. We find no basis whatsoever for the interpretation of "pseudotubuli" as being mostly atrophic liver cell trabeculae, which have been preserved and remained lying in the same place (Herxheimer and Tholldte), or as being formed by pressure atrophy of liver epithelia as a result of new reticular cell formation of intra-acinic capillaries (Coronini), although our sections show that "pseudotubuli" and extensive parenchyma atrophies are present side by side. It is true that "pseudotubuli" are found in the area of collapsed liver cell trabeculae, but always only at the periphery of liver lobules and nowhere in the central or intermediate lobular areas; transformations of atrophied liver cell trabeculae to ring-shaped structures provided with a lumen are found, it is true, but these are clearly different from bile duct proliferations, as far as their cellular arrangement and nuclear position are concerned. Perhaps the changes observed in our case are still too recent to allow a sufficient intralobular reticular

cell proliferation as understood by Coronini.

Morphologically, atrophied or regenerated liver cells and "pseudotubulus" epithelia can, in general, be clearly differentiated on the basis of their specific nuclear and plasma structures and their staining properties. The plasma body of bile duct epithelia is smaller, less eosinophile, lighter and has a finer structure than that of liver epithelia; it has mostly a cubic to spindle shape and, when stained with Azan, differs in its paler grey color from the more violet-red colored and coarse-grained plasma of liver epithelia. Whereas the adjacent liver cells often contain drop-shaped fatty infiltrations, the epithelia of biliary tract proliferations are completely free of such deposits, and they also show nowhere the presence of lipofuscin; their nuclei have a difinite longitudinal-oval shape, are glass-clear, transparent, and hypochromatic (low in chromatin), and contain several, but only sparse nucleoli. At the nuclear periphery, the tender and homogenous chromatin network becomes more compact (dense) and forms a sharply outlined border. On the contrary, the nuclei of liver cells are mostly round, less transparent and exhibit a different kind of chromatin structure.

We believe, therefore, that we can agree with Hammar's opinion stating that liver cells and bile ducts, in their substitutive formation, multiply (increase) separately in accordance with fetal formation processes; however, we cannot agree with Hammar when he states that all transition forms between liver cells and bile duct epithelia are completely absent (lacking). Stimulated, at first atrophied and then regenerated liver cells located in the outer peripheral lobular zone can be differentiated only with great difficulty at isolated spots from the broadly adjacent "pseudotubulus" epithelia and are, to a large extent, quite similar. We are unable,

however, to deduce the formation of "pseudotubuli" from liver cells on the basis of these very infrequent location spots, as compared to the amount of "pseudotubuli" present. We believe, rather, that a simultaneous and joint (combined) proliferation of liver cells and intermediate parts of perilobular, septal bile ducts is taking place. Criteria which would indicate a transformation of liver cells to "pseudotubuli" or vice-versa cannot be found anywhere.

Signs of a resorption of the destroyed tissue, in the form of an extensive resorptive inflammation, are particularly clear. Compact (dense) round cell infiltrations of lymphocytes, plasma cells and lobed-nuclear leucocytes surround, in a belt-like fashion, the parenchyma necroses and are still found extensively in the expanded (dilated) periportal fields between healthy liver lobules. The dilated capillaries of liver lobules adjacent to the necroses containing large numbers of nucleated blood elements. The Kupffer's cells within the parenchyma necroses, as well as the proliferated connective tissue cells and intralobular capillary endothelia located in their vicinity, contain large amounts of phagocytized decomposition pigment, and the latter are also rich in iron-containing pigment from capillary hemorrhages. Leucocytes are also found, along with pigment, in the bile ducts, as the expression of an excretory cholangitis.

As signs of a direct irritation of the mesenchyma, we find an increase (multiplication), swelling, rounding and excoriation (peeling) of capillary endothelia within the parenchyma necroses, and also deep inside the adjacent preserved liver lobules, in the sense of a clearly expressed, intralobular reticulo-endothelial reaction, as well as a strong proliferation of connective tissue cells in the area of adjacent periportal fields, primarily in the

form of fibroblasts. The latter have started to form new collagenous fibrils and have already caused, in some spots, some pressure damage in neighboring liver cell trabeculae. A slight thickening and collagenous impregnation of the reticular fiber network, in the sense of a fibrillary sclerosis, can be detected within the damaged parenchyma portions. To what extent a relationship to fiber-forming cells is present in this case cannot be decided in view of the simultaneous strong endothelium irritation.

We are now confronted with the question of the formation of liver cirrhosis following injury by acetic acid. A cirrhosis after chronic liver atrophy can be expected, at the earliest, after 4 months (Rossle), and thus far our case, in which the beginning of injury occurred 8 days prior to death, offers little hope. Nevertheless, we believe that we can make some contribution to the genesis of liver cirrhosis in view of the peculiar nature of certain changes observed in our case.

At first, we must point out that our case is characterized by the presence of focal, infarct-like injuries of the liver. Partial cirrhoses have been described in different ways, however, those by Rossle, among others, with a typical Laennec's protuberance (nodulation) and a depth extension of about 1 cm, as the probable consequence of a rougher vascular stenosis. While Kretz, Joannovicz and Fischler consider that an intermittent parenchyma injury is the primary cause of liver cirrhosis, an opposing fact is that extensive parenchyma disorders damage can also be completely regenerated (Jaffe) without any signs of cirrhosis formation. Liver cirrhosis requires a toxic action directed at the same time against the liver epithelium and mesenchyma, and also a slow (lingering) inflammation along with parenchyma losses. In our case, the strong resorptive inflammation is supplemented

by a direct irritation of the mesenchyma with proliferations of capillary endothelia and periportal connective tissue cells, as well as by extensive disruptions of circulatory conditions in the region of intra- and perilobular capillaries, especially promoted by the scattered and blotched nature of parenchyma necroses, which fragment (dissect) the liver lobules. According to Rossle, injury of the vascular network is the leading factor of the cirrhotic process, and the connective tissue proliferation and parenchyma degeneration are its consequences. Jaffe sees a significant moment in the increased hemocytolysis, although Rossle denies that strictly erythrocytolytic poisons possess any kind of cirrhogenic action. Only a combined injury of capillary walls and liver cells makes it possible for blood poisons to induce a scar-forming hepatitis, and tests with hemoglobin solutions (Jaffe), hemolytic poisons (Paerce) and fatty acids (Boix) must also be interpreted in this sense. In order to induce a true partial cirrhosis, however, the harmful product must be able to exert a continuous and repeated action for some time. This continuous action is assured, in our case, by the extensive thromboses in the fine ramifications of the portal vein.

Finally, the liver cirrhosis picture also includes an extensive scar formation (cicatrisation), resulting from deficient tissue restoration. As the first signs of such a cicatrisation, we find a tender pericapillary and also a stronger periportal proliferation of the connective tissue in the area of damaged liver sectors. In some spots, intralobular capillaries are obliterated and are transformed into fine connective tissue strands; in the final account, endothelia must be considered as suppliers of connective tissue. In this manner, we have confirmed the findings reported by Pearce, who observed the neoformation of intercellular fibrils already on the 5th

day and of blood vessels on the 11th day after injections of hemolytic immune sera.

As a result of the scarred shrinkage areas, the liver capsule exhibits small, funnel-shaped retractions at the point where the tissue necroses come into contact with the surface. Extensive bile duct proliferations, as well as the non-regenerating "pseudolobuli", complete the picture of a cirrhosis in its early stages.

On the basis of the histological picture obtained, we are entitled to assume that the flooding of acetic acid through the portal vein causes an injury of the hepatic tissue as a whole; for this reason, acetic acid must be included in the large group of cirrhosis-inducing poisons.

Summary

A case of acute peroral poisoning by highly-concentrated acetic acid is reported, in which the patient died on the 8th day; the autopsy disclosed, along with high-degree caustic burns of the digestive tract, extensive thromboses of stomach, omentum and splenic veins, as well as peripheral portal vein ramifications with map-like anemic infarct necroses in the liver and spleen, which are considered as direct consequences of an overflooding of the blood by acetic acid.

The liver injuries resemble most closely those observed in eclampsia, and are characterized by deep-seated circulatory disorders, accompanied by thromboses, in the area of portal vein branches and intralobular capillaries, by necroses and atrophy of the parenchyma, and also by an irritation of the mesenchyma with a strong reticulo-endothelial reaction and extensive round cell infiltrations.

To these injuries, the hepatic tissue responds early by regeneration of liver epithelia and proliferations of the periportal and intralobular connective tissue and of bile ducts.

Attempts are made to interpret the origin of the numerous so-called pseudotubuli formed as a result of these injuries.

On the basis of a precise knowledge about the duration of the action of the toxic product, new criteria are obtained for the timely measurement of the beginning of liver regeneration and cirrhotic scar formation (cicatrisation).

Acetic acid belongs to the group of cirrhosis-inducing poisons in view of its simultaneously injuring and irritating action on liver tissue.

Bibliography

D'Amato, L.: Virchows Arch. 157, 435 (1907). — Ehrmann, G. v.: Klin. Wschr. 1927 I, 778. — Foix, E. Ch.: Thèse de Paris. 1894. — Borutta: Berl. Klin. Wschr. 1929 II, 1752. — Cameron, G. R., W. A. E. Kapusarewicz and J. C. Thomas: J. Path. a. Bacter. 44, 937 (1937). — Cordoni, C.: Wiener med. Wschr. 1935 V, 231. — Dorawski, L. u. Fr. Roeder: Virchows Arch. 292, 255 (1934). — Ebner: Ärztl. Sachverstztg. 1913. — Fischer, E.: Dtsch. Arch. klin. Med. 93, 427 (1908). — Fraenz: Friedreichs Bl. gerichtl. Med. 60 (1909); 61 (1910). — Gumprecht, H.: Frühners Slg Vergift.fälle 1, 27. — Hanzal, J. A.: Z. mikrosk.-anat. Forsch. 5, 50 (1926). — Heine, C.: Virchows Arch. 41, 24 (1867). — Hueper, G. u. Tedrowa M.: In Henke-Lubarschs Handbuch der speziellen pathologischen Anatomie, Bd. V/1. 1933. — Jaffé, R. H.: Frankf. Z. Path. 24, 241 (1920). — Jessenovec, G.: Wiener klin. Wschr. 1934, 757. — Dr. Jessner und Jost: Mitt. Grenzgeb. Med. u. Chir. 24, 109 (1911). — Jost, H.: In Handbuch der normalen und pathologischen Physiologie, Bd. V. 1928. — Kretz, R.: Virchows Arch. 222, 50 (1916). — Loos u. Meyers: Virchows Arch. 210, 78 (1910). — Max, M.: Prag. med. Wschr. 1914 II, Nr 29 u. 30. — Meyer, E.: In Handbuch der normalen und pathologischen Physiologie, Bd. VI/1. 1925. — Parsons, J. u. A. Hanson: C. r. Soc. Biol. 1937, 539. — Pearce, R. M.: J. exper. Med. (Am.) 8, 61 (1906). — Pepper, L.: In Henke-Lubarschs Handbuch der speziellen pathologischen Anatomie, Bd. X. 1930. — Pertz, W.: In v. Mollendorffs Handbuch der mikroskopischen Anatomie des Menschen, Bd. V/2. 1932. — Raesebeck, G.: Untersuchungen über die Widerstandsfähigkeit der Leber gegen Gifte. Leipzig 1927. — Robinson, R. A.: Verh. dtsch. path. Ges. 1912. — Rössle, R.: In Henke-Lubarschs Handbuch der speziellen pathologischen Anatomie, Bd. V/1. 1934. — Schwarz, E.: Ärztl. Sachverstztg. 1902, 15. — Schmorl, A.: Vjschr. gerichtl. u. öff. Med. 55, 157 (1918). — Simmonds, G.: Verhdl. dtsch. path. Ges. 1912. — Schulze, O. u. L. R. Müller: Dtsch. Arch. klin. Med. 78, 544 (1903). — Zimmermann, O.: Z. klin. Med. 1938, 453. — Vecchiotti: Riforma med. 1914, Nr 4.

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Über die Leberveränderungen bei einer Essigsäure-Vergiftung und ihre Bedeutung für die zeitliche Bemessung von Leberregeneration und cirrhotischer Narbenbildung.

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Vergiftungen mit konzentrierter Essigsäure (Essigessenz) kamen um die Jahrhundertwende häufig zur Beobachtung; sie waren meist Folge von Unglücksfällen. Strenge gesetzliche Bestimmungen dämmten die rasch angewachsene Zahl der Todesfälle bald ein; seitdem weisen die Statistiken nur noch selten derartige Vergiftungen auf.

Das klinische und morphologische Bild der Essigsäurevergiftung ist durch zahlreiche Arbeiten aus jener Zeit, insbesondere durch die Zusammenfassungen von Schäffer, Franz, Marx und Schibkow eingehend beleuchtet und weitgehend geklärt worden; es soll hier nicht wiederholt werden. Kurz ergibt sich folgendes:

Die Vergiftung kann vom Verdauungskanal aus, durch Injektion, durch Aspiration, wie auch von der Haut aus erfolgen und den Tod schon in wenigen Tagen herbeiführen. Die Wirkung der Essigsäure ist eine zweifache:

Einmal eine *ätzende*, indem sie durch Eiweißkoagulation und besondere lipoidlösliche Eigenschaften rasch tiefgreifende Veränderungen an den Geweben setzt, zum andern eine *hämolytische*, indem sie nach Diffusion durch die Gefäßwandungen eine direkte Schädigung des Blutes hervorruft. Im Vordergrund steht dabei eine schwere Schädigung der Erythrocyten auf Grund der hohen Affinität der Essigsäure zu deren Lipoidhülle. Die Erythrocyten schrumpfen, blassen ab und werden schließlich gänzlich zerstört.

In den Gefäßen kommt es zu ausgedehnten *Thrombosierungen*, sowie zur Braunfärbung des unmittelbar berührten Blutes. Das flüssige Blut wird lackfarben und enthält spektroskopisch nachweisbares Hämoglobin.

Die starke Erythrolyse führt über eine *Hämatinämie* zum echten hämolytischen (anhepatischen) *Ikterus* („Hämatinikterus", nicht „Bilirubinikterus"). In schneller Folge stellen sich Veränderungen an

der Milz durch Hämoglobinimbibition, an den Nieren durch Hämoglobincylinder und -infarzierungen mit Epithelnekrosen sowie eine *Hämoglobinurie* ein. Durch Säurewirkung kommt es zum Alkalientzug des Blutes und damit zu allgemeiner Acidität; die Verätzung des Magen-Darmtraktes führt zu starkem Blutverlust; Viskositätsveränderungen des Blutes bedingen Zirkulationsstörungen und Erschwerung der Herztätigkeit; die Blutungszeit wird verlängert.

Infolge der Einwirkung von Essigsäuredämpfen oder Säureaspiration finden sich fast stets Schädigungen des Respirationstraktes, auch dann, wenn die Veränderungen im Verdauungskanal auffallend gering geblieben sind. In über der Hälfte der tödlichen Vergiftungen kommt es zu *hämorrhagischen Pneumonien*. Bei Tieren ist bereits die Einatmung verdampfender Essigsäure tödlich.

So können die verschiedensten Störungen schließlich den Tod herbeiführen. Unser Fall einer tödlichen Vergiftung reiht sich diesen Befunden zwanglos ein. Was ihn besonders hervorzuheben rechtfertigt, sind eigenartige Veränderungen an Leber und Milz in Gestalt umschriebener, infarktähnlicher anämischer Nekrosen im Gefolge ausgedehnter Gefäßthrombosierungen, wobei es in der Leber zu beginnenden reparativen und cirrhoseartigen Reaktionen gekommen war. Die Ergebnisse der Tierexperimente mit Essigsäure zur Erzeugung von Lebercirrhose sind sehr widersprechend; unserem Fall kommt hier besondere Bedeutung zu als einer Art Experiment am Menschen, das klinisch und pathologisch-anatomisch genau zu registrieren war:

41jähriger Bufettier, der vor 1 Jahr in sehr elendem Zustand und auf Krücken wegen einer Beinverletzung aus russischer Kriegsgefangenschaft zurückgekehrt war. Er hatte wegen Typhus abdominalis 7 Wochen stationär gelegen; trotz anfänglicher guter Erholung kam es bei Alkohol- und Nikotinabusus langsam zu zunehmendem Kräfteverfall. Am Tage nach der Entlassung trank Patient in selbstmörderischer Absicht 200 cm³ einer 60%igen Essigsäurelösung. Sofort setzte schwere Übelkeit mit Schmerzen im Oberbauch und blutigem Erbrechen ein; 6 Stunden später waren Stuhl und Urin blutig. Der hinzugezogene Arzt vermutete zunächst ein blutendes Magengeschwür. Erst nach 30 Stunden erfolgte wegen zunehmender Schmerzen die Einweisung in ein Krankenhaus.

Bei der Aufnahme fanden sich kleine verschorfte Wunden am Mundwinkel und an hinteren Zungenteilen. Zu quälenden Schmerzen gesellte sich allmählich brennendes Durstgefühl. Beim mehrmaligen Versuch, eine Nährsonde anzulegen, stieß sich stets bei 20—25 cm ein Stop. Der Kreislauf verlangte dauernde Stützung. Nach 2 Tagen hatte der Brechreiz nachgelassen, aber der brennende Durst trotz Traubenzucker- und Kochsalzinfusionen zugenommen. Die Temperatur lag über 38°, der Puls war anhaltend hoch um 100—130. Blutsenkungsreaktion 25. Im Harn fanden sich Spuren von Eiweiß, im Sediment neben reichlich Leukocyten einige Erythrocyten; Urobilin, Uribilinogen und Bilirubin normal.

Nach 5 Tagen erfolgte Verlegung auf die laryngologische Klinik der Charité. Im Vordergrund der Erkrankung stand nun ganz der sehr hochgradig beeinträchtigte Kreislauf. Die Haut war ausgesprochen blaß; Patient konnte nicht sprechen und war stark kurzatmig; sein Zustand ähnelte dem im Koma. Der Puls

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war fadenförmig und lag um 160, die Temperaturen reichten fast an 40° C. Die gesamte Schleimhaut von Mund, Gaumen und Gaumensegel, sowie der Nase war hochgradig eingetrocknet und mit harten Krusten belegt. Der Versuch, Patienten selbständig schlucken zu lassen, gelang unter beträchtlichen Beschwerden. Am nächsten Tag wurden Mund- und Nasenhöhle von Borken gereinigt und mit Borglycerin ausgepinselt. Das Anlegen eines Nährschlauches gelang jetzt ohne Mühe. Herz- und Kreislaufunterstützung wie Infusionen blieben jedoch nur von vorübergehendem Erfolg; Patient starb am 8. Tage nach der Vergiftung.

Sektionsbefund. Starker Schwund der Fettpolster. Deutlich ausgeprägte Totenstarre. Auffallend blasse, aber nicht ikterische Haut, doch gelbliche Skleren. Um Mund- und Nasenöffnung ausgedehnte grau-schwarze Borken; Zahnfleisch, Mundschleimhaut und Zunge mit schmutziggrauem bis bräunlichem Schorf bedeckt. Rachen stark gerötet; Larynx und Epiglottis mässig ödematös geschwollen. Speiseröhre deutlich erweitert, ihre Schleimhaut fleckig bis streifig mit grau- bis rotbraunem, mattem Schorf belegt, besonders in den unteren Abschnitten; stellenweise fehlt die Schleimhaut ganz. Magen stark kontrahiert; Serosa schiefergrau bis schwarz, matt glänzend; die Wandung geschwollen, starr und trocken. Im Magen nur wenig dickflüssiger, rotbrauner und teerartiger Inhalt von kennzeichnend aromatisch saurem Geruch. Schleimhaut breit und wulstartig gefältelt, ungleichmäßig braun- bis düster schwarzbraun-rot verfärbt; sie ist im Pylorusmagen ausgedehnt nektrotisch, mürbe, blutig durchtränkt und läßt sich von der Unterlage breit abheben; in der Cardia zeigt sie sich besser erhalten, unregelmäßig gehöckert, graubraun. Der stark kontrahierte Pylorus trennt sich scharf gegen das Duodenum ab, dessen Schleimhaut nur auffallend geringe, kleinfleckige Blutungen aufweist.

Großes Netz schmutzig gelb-braun verfärbt, sein Fettgewebe weitgehend geschwunden. Die Venen der Magenwand und der anliegenden Teile des Netzes und Gekröses auffallend weit, stark hervortretend und prall mit rotbraunen bis schwärzlichen, geronnenen und feingeriffelten Blutmassen ausgefüllt. Die untere Hohlvene und die unteren Abschnitte der Milzvene enthalten flüssiges, lackfarbenes Blut.

Im oberen Teil der Milzvene bis hinauf in die feineren Äste zart geriffelte und der Wand teils anklebende, teils festanhaftende, dunkelrotbraune, stellenweise weißlich gefleckte Blutpfröpfe, die die Lichtung vollständig verlegen. Milz gering vergrößert, an der Oberfläche wie auf dem Schnitt landkartenartig, teils gelb-grau, teils düster blau-rot gefleckt; Pulpa mäßig gelockert und abstreifbar.

Leber gering vergrößert; Oberfläche glatt; durch die Kapsel des linken und rechten Lappens schimmern einzelne, bis fünfmarkstückgroße, umschriebene, unregelmäßig fleckige Parenchymteile in hell-gelblicher bis blaß-bräunlicher Farbe; auf dem Anschnitt erstrecken sie sich keilartig und infarktähnlich gegen den Hilus und heben sich durch eine mattgelblich-graue, lehmige Farbe und stark verwischte Läppchenzeichnung vom übrigen mehr rötlichen, etwas trübglänzenden, blut- und saftreichen Parenchym deutlich ab. Die zugehörigen kleinen Pfortaderäste sind durch dunkelbräunliche, mürbe Thromben verschlossen, die sich stellenweise bis in mittelgroße Äste hinein verfolgen lassen; das Hauptrohr der Pfortader enthält nur flüssiges Blut. Weder hier noch an den Venen, Arterien oder Gallenwegen sind mit bloßem Auge krankhafte Veränderungen erkennbar.

Beide Nieren mäßig geschwollen, feucht glänzend, blutreich; Rinde blaßbräunlich und leicht getrübt; Pyramiden düsterrot mit geringer radiärer braunschwarzer Strichelung.

Die Schleimhaut der Harnblase zeigt inmitten einer umschriebenen, hochgradigen Rötung einen schmutzig-grauen Defekt.

Dick- und Dünndarmserosa fleckig violettgrau verfärbt. Dickdarm sehr weit, die Wandung seiner oberen Abschnitte ödematös geschwollen, fleckig-düsterrot; seine Schleimhaut ausgedehnt defekt und von graurotem Schorf bedeckt. Die Schleimhaut des unteren Ileums hochgradig gerötet, die Peyerschen Plaques geschwollen und streifenartig graurot imbibiert.

Trachea und Bronchien enthalten reichlich zähen, bräunlich-rötlichen Schleim; ihre Schleimhaut etwas geschwollen und gleichmäßig stark gerötet. Beide Lungenunterlappen groß und schwer und von unregelmäßigen, stellenweise flächenhaften, rotgrauen und feingekörnten pneumonischen Herden durchsetzt. Pleura pulmonalis über beide Unterlappen, besonders rechts, matt und mit frischen fibrinösen Auflagerungen versehen.

Sektionsdiagnose (36/47): Verätzung des Magens durch Essigsäurevergiftung. Hochgradige, hämorrhagisch nekrotisierende Gastritis. Mäßige Schorfbildung mit oberflächlicher Geschwürsbildung sowie Erweiterung der Speiseröhre. Thrombose der V. lienalis mit hierdurch bedingter infarktähnlicher Nekrose in der Milz; Thrombose von Pfortaderästen mit Lebernekrosen; Thrombosen von Netz, Magen- und Gekrösevenen. Ausgedehnte hämorrhagische Enteritis; hochgradige verschorfende Colitis. Pharyngitis. Starke Tracheobronchitis. Fleckige croupöse Pneumonie beider Lungenunterlappen, im rechten konfluierend; beiderseitige trockene Pleuritis; hämorrhagisches Ödem der übrigen Lungenlappen. Umschriebene Nekrose der Harnblasenschleimhaut. Cyanose und Trübung der Nieren. Dilatation des rechten Herzens; braune Degeneration des Myokards. Geringe Narbenbildung der Aortenwurzel; Fibrosis testis. Chronische Tonsillitis. Ödem der Hirnhäute; Blutungen in der Leptomenix. Länge 176 cm, Gewicht 51 kg. Leber 1990 g, Milz 210 g.

Mikroskopischer Befund. 1. Leber. Der Schnitt durch einen der infarktartigen Leberbezirke zeigt peripher von thrombotisch verschlossenen, kleineren Pfortaderästen unscharf begrenzte, fleckig-wolkige Parenchymnekrosen in landkartenartig gruppierten Herden. Sie sind von der Leberoberfläche durch einen wechselnd breiten Saum erhaltenen Parenchyms getrennt (Abb. 1). Wo sie sich der Leberkapsel zungenartig nähern, ist diese kernreicher und narbig eingezogen.

Die Blutpfröpfe der Pfortaderäste verschließen die Gefäßlichtung vollständig und zeigen eine Schichtung von Erythrocyten, Fibrin mit Thrombocyten und zelligen Elementen gegeneinander, was auf ihre allmähliche Entstehung nach vorangegangener Stase schließen läßt. An einzelnen Stellen ist die Gefäßintima aufgelockert und vom Endothel entkleidet; bindegewebige Zellelemente beginnen hier die Organisation des Thrombus. Arterien- und Lebervenen sind durchweg frei von pathologischen Veränderungen; die gröberen Gallenwege enthalten in ihren äußeren Wandschichten stellenweise geringe herdförmige Rundzelleninfiltrationen.

Die wolkigen, von erhaltenen Parenchymteilen untermischten *Gewebsnekrosen* bestehen aus mosaikartig zusammengewürfelten und engangrenzenden, gegeneinander abgekanteten, kernlosen Leberzellschollen (Abb. 2), deren Zellmembran starke Schädigungen aufweist; ihr Protoplasma ist blaß eosinophil, verwaschen feinkörnt, kaum transparent und durchsetzt von einzelnen nicht sudanophilen Vacuolen. Viele Schollen zeigen eine wechselnd starke, diffuse gallige Durchtränkung, manche ein feinkörniges, gelb-bräunliches Zerfallspigment. Die Läppchenstruktur wird nur noch durch das durchweg gut erhaltene Gitterfasergerüst dargestellt, das die einzelnen Zellschollen als feines, durch Azan kräftig gefärbtes Netzwerk umspinnt; es erscheint gegenüber dem in gesunden Läppchen eher vermehrt und vergröbert und ist besonders deutlich um die leeren Zentralvenen, die mit ihren verdickten und aufgelockerten Gefäßwandungen wie isoliert im Gewebe zu liegen scheinen (Abb. 1).

Von einer Capillaranordnung ist nichts mehr zu erkennen; nur hier und da durchziehen kleine, einzeln stehende und schwach blutgefüllte Capillaren das untergegangene Parenchym. Die meisten Capillaren sind kollabiert und täuschen so eine Vermehrung des Gittergerüstes vor. Demgegenüber sind die Capillarendothelien überall deutlich, ja sie scheinen stellenweise vermehrt und werden nur dort selten, wo eine stärkere Verwischung der Leberzellgrenzen die fortgeschrittene Nekrose anzeigt. Hier und da umschließen sie mit langen, flügelartigen

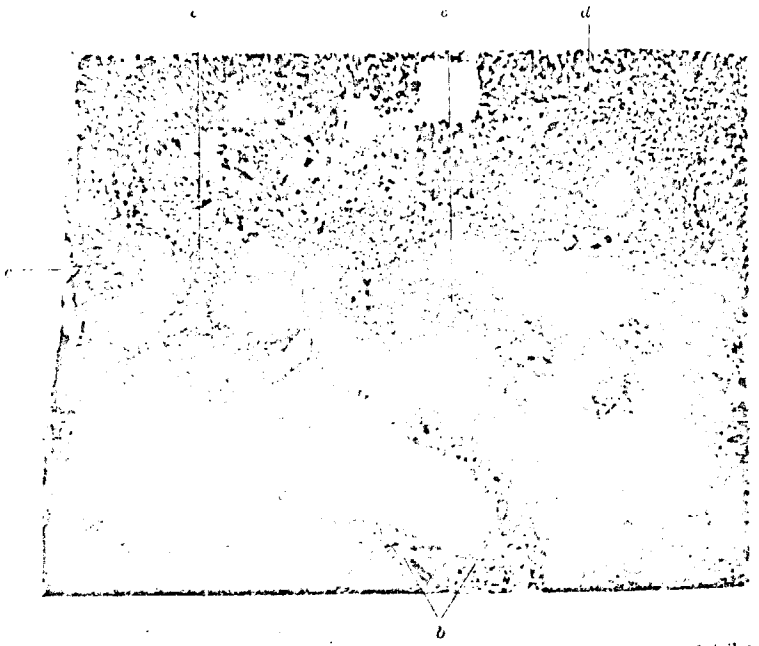

Abb. 1. Teilübersichtsbild. Fleckige Nekrosen des Lebergewebes (*a*) mit noch teilweise erhaltener Gewebsstruktur und Verbreiterung des Interstitiums (*b*). Geringe narbige Einziehungen der Leberkapsel (*c*). Starke Erweiterung der benachbarten intralobulären Capillaren (*d*). Zentralvenen (*e*). Häm.-Eosin. Lupenvergrößerung.

Fortsätzen kleine Fragmente von nekrotischen Leberepithelien; andere sind spindelig ausgezogen nach Art der Fibrocyten und haben intraacinär mit der Bildung eines zarten, kollagenen Fibrillennetzes begonnen, ohne daß ein Zusammenhang mit dem periportalen Bindegewebe besteht. Ihr Zelleib ist meist deutlich geschwollen und vollgepfropft mit feinen, gelblichen, nur teilweise eisenhaltigen Pigmentkörnchen. Deutlicher noch als der Leib ist zuweilen der Kern geschwollen. Durch Abrundung der Kernpole nimmt er eine mehr längliche ovale Form an und zeigt feine, mehr randständige Chromatinstrukturen. Sie erinnern diese Zellen nur noch zum kleineren Teil an Endothelien und lassen sich von Fibroblasten kaum noch unterscheiden.

Die Nekrose der Leberzellen ist nicht an die Läppchenstruktur gebunden; sie hat die Leberläppchen in wechselnder und unregelmäßiger Ausdehnung ergriffen. Viele sind in schwankender Breite sektorartig, andere tangential befallen, manche vor oder hinter der Zentralvene glatt halbiert; wieder von anderen finden sich nur noch einzelne Balkenteile (Abb. 2 und 3). Alleinige Nekrosen um Zentralvenen oder erhaltene Leberzellen in ringartiger Anordnung um Glissonsche Felder sind nirgends nachweisbar; alle Untergänge zeigen breite Verbindung zur Läppchenperipherie. Der Ausgangspunkt der Gewebsschädigung ist daher in den periportalen Feldern zu suchen.

Der Übergang zwischen nekrotischem und erhaltenem Leberepithel ist nur stellenweise verhältnismäßig scharf. Eine oft nur einreihige Schicht kollabierter

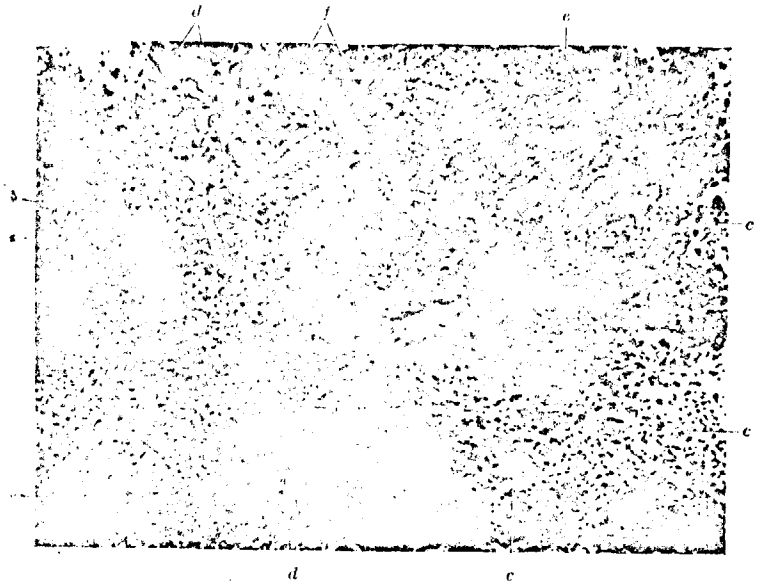

Abb. 2. Grenzgebiet zwischen Nekrose und erhaltenem Leberparenchym. Wucherung der Kupfferschen Sternzellen im Bereich der Nekrosen (*a*). Unregelmäßige, vorwiegend tiefere Zerstörung angrenzender Leberläppchen. Resorptive und proliferative Entzündung im Bereich der Grenzscheide mit Wucherung des Mesenchyms (*b*) und der Gallengänge (*c*), sowie starker rundzelliger Infiltration vor den dissoziierten Ausläufern der Leberzellbalken (*d*). Atrophie der erhaltenen Leberzellen (*e*) mit Erweiterung der Capillaren; Vermehrung der kernhaltigen Blutzellen innerhalb der Capillaren und Zentralvenen (*f*); starke Retikuloendothelreaktion. Häm.-Eosin. 320fach.

und nekrotischer Leberzellen mit grobtropfigen Fetteinlagerungen im Protoplasma, trüber Körnelung und vacuoliger Degeneration, sowie pyknotischen Kernen, trennt die beiden Schichten und ist bandartig durchsetzt von dichtgelagerten, stark pigmentbeladenen, fibroblastenähnlichen Zellen (Abb. 3).

Nicht aber läuft das erhaltene Leberparenchym allmählich in die Nekrosen aus (Abb. 2). Die Leberzellbalken verschmälern sich zunehmend, das Zellplasma, meist stärker eosinophil, blaßt ab; die Kerne werden kleiner, ihr Chromatin verdichtet sich. Das pericelluläre Gitterfasergerüst tritt deutlicher hervor, spaltet sich auf und verliert sich unter Aufgabe des Bälkchengefüges zu einem weiten und feinmaschigen Netz, in dem wie aufgehängt anfänglich noch Leberzellreste mit spärlichem, blaßgrauem, oft vacuoligem und zipfelig ausgezogenem Protoplasma erkennbar sind (Abb. 4). In den Maschen des Netzes haben sich die durch Verschmälerung der Zellbalken entsprechend verbreiterten intralobulären Capillaren seenartig ausgedehnt und mit Erythrocyten aufgefüllt. Dabei ist stellenweise zu Einrissen der Capillarwand mit Extravasaten gekommen.

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Dementsprechend tragen die stark gewucherten Capillarendothelien reichlich eisenhaltiges Pigment, und das ist zuweilen das einzige Hilfsmittel, sie von den atrophischen Leberepithelien zu unterscheiden.

Aber nicht alle randständigen Leberepithelien gehen zugrunde. Viele zeigen deutlich den Versuch zur *Regeneration*. So liegen vor den manchmal plump und kolbenartig aufgetriebenen, stärker gewundenen Ausläufern der Leberzellbalken

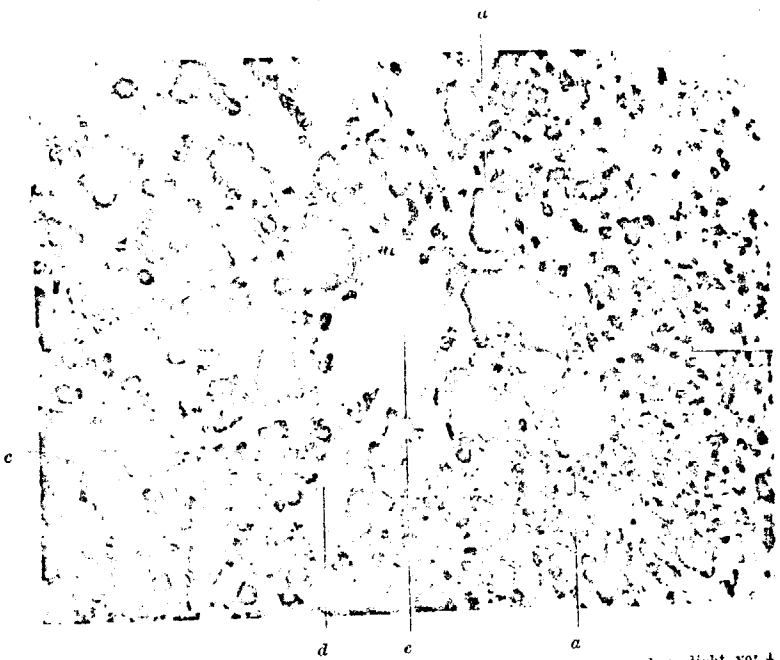

Abb. 3. Zentrum eines Leberläppchens mit teilweisem, das Läppchen dicht vor der Zentralvene quer halbierendem Parenchymuntergang. Atrophie, Nekrobiose und grob- tropfige Verfettung der Leberzellen im Grenzgebiet (*a*). Starke, ungeordnete Wucherung der Capillarendothelien im Bereich der ausgefallenen Parenchymzellen (*b*). Dissoziierte und feintropfige Verfettung der erhaltenen Leberzellbalken (*c*) mit Erweiterung der intralobulären Capillaren (*d*) und der Zentralvene (*e*). Sudan. 400fach.

(Abb. 5) abgeschnürte Gruppen von Leberzellen oder einzeln verstreut abgerundete Leberepithelien, deren Plasmaleib sich stark aufgetrieben, aber gleichmäßig feinkörnig und tief eosinophil darstellt und galliges Pigment gebildet hat. Ihre Zellgrenzen sind scharf gezeichnet, die Kerne klar und durchweg vergrößert, zuweilen riesenartig geschwollen und dann vacuolig gebläht. Häufig finden sich zwei mehrere Kerne in einem gemeinsamen Plasmaleib als Zeichen amitotischer Zellteilung. Mitotische Kernfiguren dagegen sind selten.

Die intralobulären Capillaren der den Nekrosen benachbarten Leberläppchen finden sich noch weit im Gesunden stark erweitert. Sie enthalten auffällig wenig Erythrocyten, und diese zeigen häufig Anisocytose und Anisochromie, sind jedoch reichlich von wolkigen feinkörnigen Eiweißmassen angefüllt, oft in Form cylinderartiger Ausgüsse. Zwischendurch eingestreut liegen zahlreiche kernhaltige Zellelemente, die sich nur zum Teil als Blutzellen, meist Leukocyten deuten lassen; zu einem großen Teil handelt es sich um gewucherte, abgerundete

und desquamierte Capillarendothelien im Sinne einer *Endothelproliferation* (Abb. 2). Die Capillarwände sind aufgelockert und stellenweise abgehoben, ihre Fibrillen oft unscharf konturiert. Pericapillär finden sich vereinzelt lymphocytenartige oder fibroblastenähnliche Zellelemente, sowie schleierartige homogene Plasmaschlieren.

Die periportalen Felder sind stark verbreitert und verbinden sich mit den zwischen und vor den kollabierten Läppchenausläufern gelegenen, zarten Endothelproliferationen zu einer die Nekrosen mantelartig einhüllenden, in seiner

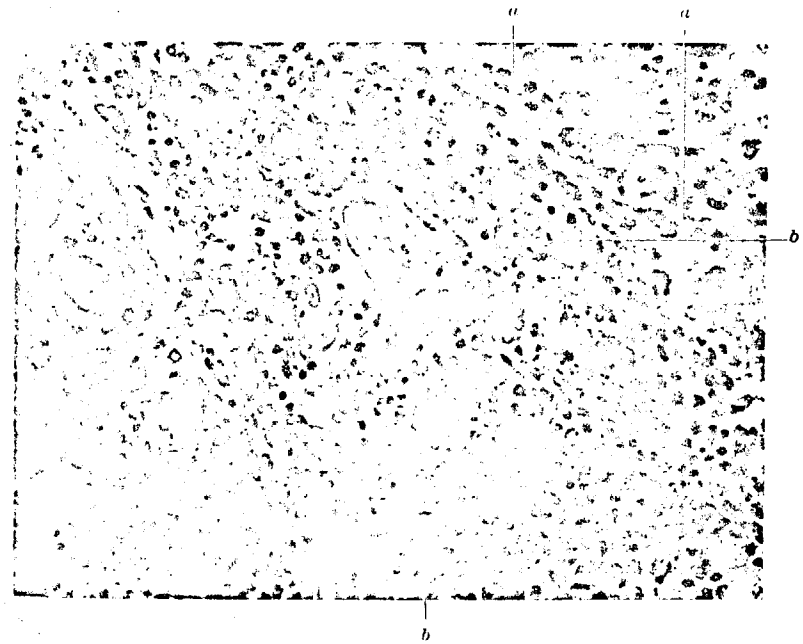

Abb. 4. Starke Wucherung des gering rundzellig infiltrierten Mesenchyms mit Verdichtung des Fasergerüstes. Gallengangswucherungen. Kollaps von Leberzellbalken (*a*) mit netzartiger Proliferation der Capillarendothelien und des Gitterfasergerüstes (*b*). Häm.-Eosin. 400fach.

Breite wechselnden, bindegewebigen *Organisationszone*. Diese besteht größtenteils aus fibroblastenartigen, weniger aus histiocytären Zellen, die mit einem meist eisenhaltigen Pigment vollbeladen sind und zeigt eine dichte Untermischung mit polymorphkernigen Leukocyten, Lymphocyten und besonders reichlich mit Plasmazellen. Zwischen den Zellen liegt ein dichtes Netz feinfibrillären Bindegewebes, das mit dem Gitterfasernetz der erhaltenen wie nekrotischen Leberteile kontinuierlich zusammenhängt und sich mit Azan kräftig blau färbt (Abb. 4). Es entspricht nur zum Teil kollabierten und aufgesplitterten Gitterfasernetzen der Leberläppchen; großenteils handelt es sich um junges kollagenes Fasergewebe. Eine dichte Durchsetzung mit zahlreichen, meist neugesproßten Capillaren vervollständigt das Bild zu dem eines typischen Granulationsgewebes.

Unter dem Druck dieses neugebildeten Gewebes zeigen einzelne angrenzende Leberläppchen Verschmälerung und parallele Schichtung von Leberzellbalken. In Verein mit der oft randständigen Lagerung der Zentralvenen in partiell

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geschädigten Leberläppchen und der angedeuteten intralobulären Bindegewebsvermehrung kommt es bereits zur Bildung von *Pseudolobuli*.

Die Endaufzweigungen der *Pfortader* in den GLISSONschen Feldern sind stark seenartig erweitert und vorwiegend mit Rundzellen, meist polymorphkernigen Leukocyten, sowie wenigen fibroblastenähnlichen Zellen prall ausgefüllt.

Die *Gallengänge* im Gebiet der periportalen Felder zeigen ausgedehnte Wucherungen. Sie tun dies meist in Gestalt kleiner, lichtungsloser, in Doppelreihen

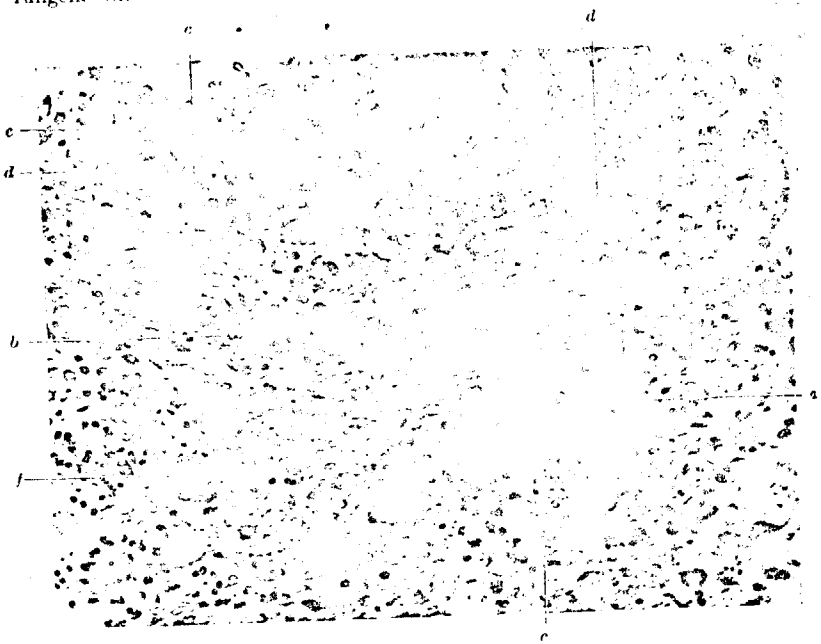

Abb. 5. Verbreitertes periportales Feld aus dem Bereich der Proliferationszone mit Wucherungen der Mesenchymzellen, Phagocytose von eisenhaltigem Pigment (*a*) und reichlichen Neusprossungen von Capillaren (*b*). Vermehrung des faserigen Bindegewebes. Atrophien (*c*) und Regenerate (*d*) der angrenzenden Leberzellen, darunter auch ringartige Wucherungen (*e*). Gallengangswucherungen (*f*). Berliner Blau-Kernechtrot. 400fach.

kettenartig angeordneter, längs- oder quergetroffener Epithelschläuche mit Neigung zu einer Ordnung um ein rundliches oder ovales Lumen. Hierbei umschließen sie häufig Kerntrümmer, polymorphkernige Leukocyten, sowie körniges Pigment. Vielerorts umschwärmen die als kleine, längliche Zellgruppen die Ausläufer der Leberzellbalken oder dringen sogar zwischen die kolbenartig hypertrophierten Balkenenden.

Noch weit abseits der Nekrosen zeigt das Leberparenchym eine deutliche Verschmälerung der Leberzellbalken; die Zentralvenen und angrenzenden Capillaren sind durchweg etwas weit und stark mit Erythrocyten gefüllt. Zentralgelegene Leberzellen enthalten reichlich braunes Abnutzungspigment und in geringer Menge auch feinste bis gröbere Fett-Tröpfchen. Auch die periportalen Felder lassen hier noch deutliche Wucherungen und starke rundzellige Infiltrationen erkennen. Die kleinen Äste der Pfortader sind weit und mit kernhaltigen Blutkörperchen reichlich angefüllt, die der Leberarterien zeigen geringe herdförmige adventitielle Infiltrate. Gallenwege sind reichlich gewuchert.

2. *Hilus lienalis*. Inmitten regelrechten Fettgewebes sind mehrere größere Venen quer getroffen, deren Lichtung durch frische, dunkelrotbraune Thromben vollständig verlegt ist. Intima rings intakt; im adventitiellen Gewebe liegen — verstreut oder in Haufen beisammen — lymphocytäre Elemente. Arterien o. B.

3. *Niere*. Glomeruli regelrecht. Die Epithelien der gewundenen Harnkanälchen zeigen in starkem Maße Veränderungen im Sinne einer trüben Schwellung und

Abb. 6. Querschnitt durch die langen HENLEschen Schleifen einer Nierenpapille. Cylinderartige Ausgüsse und Erweiterungen der Harnkanälchen durch Hämoglobin (Hämoglobinurie). Ödem des Interstitiums. Lepehne. 320fach.

enthalten stellenweise feintropfige Fetteinlagerungen; ihre Kerne sind oft nekrotisch. An den Schleifen, Schaltstücken und Sammelröhren ist die Epithelschädigung wesentlich geringer. Einzelne Epithelien der Hauptstücke wie auch abgeschilferte, kernlose Epithelien enthalten manchmal rotbräunliche, körnige Plasmaeinschlüsse. In der Lichtung von Schleifen, Schaltstücken und besonders von Sammelrohren sind Ballen gröberer, hellbräunlicher Granula gelegen, häufig vermengt von Epithelleichen und dann nach Art cylindrischer Ausgüsse (Abb. 6). Sie färben sich nach LEPEHNE dunkelbräunlich, ähnlich dem Blut, und mit Kresylviolett schwach-grünlich. Durch Erweiterung derartiger Harnkanälchen und Abplattung ihrer Epithelien täuschen sie zuweilen blutgefüllte Gefäße vor. Auch Zellenachbarten Epithelien selbst tragen häufig feine körnige, schwarzbräunliche Protoplasmaeinschlüsse. Im Bereich der Markpapillen lassen sich im Interstitium mit Berliner Blau ganz vereinzelt tiefblaue Granula nachweisen. Einzelne Schleifen enthalten auch vereinzelte Granulocyten. Das Gefäßsystem zeigt eine starke Blutstauung.

Der Übertritt der Essigsäure in den Kreislauf erfolgt hauptsächlich im Magen. Ein Krampf des Pylorus hindert die Essigsäure am weiteren Vordringen und verursacht ein längeres Verweilen. Die

Stärke der Durchätzung der Magenwand und damit die Stärke der Vergiftung steht in direkter Abhängigkeit zur Menge und Konzentration der zugeführten Lösung, sowie zum momentanen Füllungszustand des Magens. Mitschling sah eine vollständige Durchtränkung der Magenwand mit Diffusion von Essigsäure in die freie Bauchhöhle. Hierbei durchdringt die Essigsäure auch die Wand der Gefäße und untermischt sich mit dem zirkulierenden Blut. Die Erythrocyten agglutinieren und veranlassen so Zirkulationsstörungen. Die hinzutretende Hämolyse bedingt mit Hilfe frei werdender, gerinnungsfördernder Stoffe ausgedehnte Thrombosierungen der Gefäße und Capillaren, sowie eine allgemeine Hämoglobinämie. Heine injizierte Hunden hochkonzentrierte Essigsäure in die Femoralvene und erzielte ausgedehnte dunkle Thrombosen von der Injektionsstelle bis in die rechte Herzkammer. Die Verstopfung der Gefäße des Magens beschleunigt den Prozeß seiner Vernichtung. Esau sah nach Essigsäurekompressen Nekrosen der Haut infolge von Capillarthrombosen auch an den Spitzen gesunder Finger.

Die Thrombosierung der Gefäße verläuft über eine Stase zu langsam, um eine erfolgreiche Abriegelung des Kreislaufs gegen die Einwirkungen der Essigsäure zu erzielen. Das periphere Blut wird sehr bald mit freiem Hämoglobin überschwemmt. Wir sehen bereits 6 Stunden nach der Vergiftung „blutigen" Harn (Hämoglobinurie). Sicherlich ist auch mit dem Auftreten von Essigsäure im peripheren Blut zu rechnen, wie der von vielen Autoren betonte eigenartige Geruch der Organe vermuten läßt. Hierfür sprechen in unserem Fall auch Art und Frische der Lungenaffektion, die neben der Aspiration von Säuredämpfen hämatogene Ursachen anzunehmen zwingen. Nach Vincenzo wird Essigsäure unter anderem durch die Bronchialschleimhaut ausgeschieden. Demgegenüber sind hämorrhagische Pneumonien bei andersartigen Hämoglobinämien nirgends besonders erwähnt. Boix beobachtete bei Kaninchen nach peroraler Zufuhr Krystallisationen von Büscheln der Essigsäure in den Venen der Leber. Ein schneller Abbau der Essigsäure im Blut erscheint unwahrscheinlich, da diese auch als normales Zersetzungsprodukt im Blut und Harn auftritt, schwer oxyabel und relativ schwer verbrennlich ist.

Die Gefäßthrombosen finden sich in den gesamten venösen Abflußbahnen des Magens, sowie in deren nächstem Einmündungsgebiet, die obere Milzvene (V. gastroepiploica sin. und Vv. gastricae breves) und die untere Pfortader (V. gastroepiploica dextra und V. coronaria ventriculi).

Die Auswahl der von der Thrombose befallenen Gefäßbezirke in der Leber und damit das Fleckige der Nekrosen steht in Abhängigkeit von den besonderen Strömungsrichtungen der innerhalb des Pfortaderstromes geschieden verlaufenden Blutsäulen der Zuflüsse, sowie von dem Umstand, daß die Leber immer nur mosaikartig in Funktion steht.

Kreislaufstörungen der Leber allein bleiben ohne Folgen für das Parenchym. Bei trunkulärer Pfortaderverstopfung übernimmt die Arteria hepatica in ausreichendem Maße die Versorgung. Andauernder Verschluß der Pfortader wirkt im Sinne einer Inaktivitätsatrophie, wie dies die Experimente von Reddingius beweisen, und verursacht unregelmäßige Bindegewebsbildung, manchmal beträchtliche Sklerose, aber mehr nach Art der Atrophie als der Cirrhose.

Schulz und Müller beobachteten nach Pfortaderthrombose eine gleichmäßige Atrophie der Leber mit interstitieller Induration, aber ohne krankhafte Veränderungen an den Leberzellen. Das fibrilläre Gewebe war sehr zellreich und enthielt außer reichlich spindeligen Kernen und Rundzellen zahlreiche Gallengangswucherungen. Demgegenüber verzeichnen Heller, Joannovicz, Köbrich und Versé Nekrosen des Parenchyms. Gruber (II), Buday (II), Loeb (II), Brie und Josselin de Jong sahen Nekrosen mit „leichter Cirrhose". Aber weder Joannovicz noch Kretz gelang die Erzeugung einer Proliferation im periportalen Bindegewebe, geschweige denn die Erzeugung cirrhotischer Veränderungen.

Infarktnekrosen der Leber entwickeln sich nur dann, wenn auch die feinsten Pfortaderverzweigungen jenseits der interlobulären arteriovenösen Anastomosen verlegt sind, bei „terminalen" Pfortaderthrombosen, da hierbei auch der arterielle Zufluß gesperrt wird.

Die rein mechanische Natur dieser Nekrosen bewies Raestrup dadurch, daß er Meerschweinchen in die Pfortader korpuskuläre Gifte in erst nach geraumer Zeit lösbarer Kapselumhüllung injizierte, so daß sich deren Wirkung sicher nicht sofort entfalten konnte; er erzielte eine Verstopfung zahlreicher Gefäße durch thrombotische Umhüllung der eingespritzten Fremdkörper und in deren Folge ausgedehnte Lebernekrosen. Ribbert sah Nekrosen nach Injektion von die Blutgefäße verlegenden Substanzen wie Knochenöl und Agar-Agarlösung in die Pfortader. Schmorl beschreibt multiple Nekrosen als Folge einer primären toxischen Thrombose der kleinsten Pfortaderverzweigungen bei der Puerperalklampsie. Sicherlich beruhen auch die Nekrosen durch Immunsera zum Teil auf der Bildung hyaliner Thromben.

Lebernekrosen als Folge einer toxischen Schädigung des Parenchyms sind vielfach beobachtet und an Tieren experimentell durch Injektionen verschiedenartigster hochkonzentrierter Gifte wie Äther, Chloroform, Tetrachlorkohlenstoff, Alkohol, Phosphor oder anderer Stoffe in die Pfortader oder Mesenterialvene erzeugt worden.

Als besondere Lebergifte haben sich alle Stoffe von schwerschädigender Wirkung auf das Blut erwiesen. Nach Jaffé sind durchweg alle Blutgifte auch als Lebergifte anzusprechen und zwar auf Grund ihrer schädigenden Wirkung auf die Erythrocyten. So erzielten Jaffé mit Hämoglobinlösungen, Joannovicz, Pearce und Wooldrige mit hämolytischen Immunseren schwere Parenchymdegenerationen. Eine toxische Parenchymschädigung nimmt Fahr auch für die Eklampsie an.

Mitteilungen über Leberveränderungen nach Essigsäurezufuhr beim Menschen sind äußerst spärlich. Zumeist wurde einzig eine akute Hyperämie der Leber vermerkt. Den einzigen, unserem vielleicht entsprechenden Fall schilderte Gerhartz sen., wenn auch leider ohne mikroskopischen Befund; er beobachtete am 3. Tag nach peroraler Essigsäurevergiftung cyanotische Infarkte der Leber. v. Bergmann sah eine klassische, durch Obduktion bestätigte, atrophische Lebercirrhose bei einem Mann, der Jahre zuvor — um sich dem Militärdienst zu entziehen — wochenlang täglich morgens nüchtern einen halben Liter Essig getrunken hatte und glaubhaft versicherte, niemals Alkohol zu sich genommen zu haben. Das „Essigtrinken" wurde früher gerne zu Abmagerungskuren benutzt; es führte meist zu chronischen Verdauungsstörungen, Magenschmerzen mit Koliken, Diarrhöen, Anämie und Kurzatmigkeit (chronischem Acetismus).

Tierexperimente zur Erzeugung von Leberschädigung durch Essigsäure sind von verschiedensten Seiten ausgeführt worden und wurden angeregt durch die Bestrebungen von Boix, die durch chronische Dyspepsien veränderten Darminhalte einzeln auf ihre toxische Wirkung auf die Leber zu prüfen. Bei Verfütterung von Butter-, Essig-, Milch- und anderen -säuren an Kaninchen erwies sich die Essigsäure vor allen anderen als besonders toxisch und reizend und weitaus als die gefährlichste; sie verursachte innerhalb von 36 Tagen starke Degenerationen der Leberepithelien, deutliche rundzellige Infiltrationen der periportalen Räume, sowie eine ausgedehnte fleckige oder streifige Sklerosierung bis zu einer der Laennecschen vergleichbaren, atrophischen Lebercirrhose. Demgegenüber erwies sich Alkohol als toxisch, aber nicht reizend auf das Lebergewebe, jedoch stark reizend auf die Magenschleimhaut. Untersuchungen über den Mageninhalt der Alkoholtrinker und Dyspeptiker, sowie das Erbrochene nach schlechter Verdauung zeigten Beimengungen von Essig- und ähnlichen Säuren in oft ansehnlicher Menge. Auch der Wein, ein häufiger Lieferant der Lebercirrhose, enthält unter anderem Essigsäure. Boix unterzieht den Begriff der „Alkohol"-Cirrhose einer scharfen Kritik und will ihn zur „dyspeptischen" Cirrhose ergänzt wissen. Er denkt dabei an eine enterogene Umwandlung des die Leber nicht reizenden Alkohols zu leberreizenden organischen Säuren, so unter anderem an einen Umbau innerhalb des Magens zu Essigsäure.

Auch v. Bergmann vermutet für die Leberveränderungen beim Alkohol ursächlich sekundäre Produkte des intermediären Abbaus und zieht hierbei auch die Essigsäure in Erwägung.

Die Ergebnisse von Boix sind viel umstritten worden, und haben Nachprüfungen nicht immer standgehalten. Josselin de Jong gelang es nicht, mit Essigsäure jene breiten Züge von interacinösem Gewebe zu erzeugen, wie sie Boix abgebildet hatte; er erhielt nur eine spärliche Cirrhosierung. Joannovicz erzielte durch Verabreichung von Essigsäure mittels Schlundsonde nur ganz leichte Infiltrationen des periportalen Bindegewebes sowie eine Atrophie der Leberzellen, jedoch ohne Zeichen schwererer Degenerationen. Vinzenzo sah Veränderungen an den Lebergefäßen bei Anwendung kleiner, aber im Laufe längerer Zeit verabreichter Säuredosen; Koelsch erhielt nach Vergiftung durch organische Acetate einzig Verfettungen in der Leber. v. Bergmanns Versuche, bei Tieren mittels Essigsäure Leberschädigungen zu setzen, blieben gänzlich ohne Erfolg.

Boix und Joannovicz betonen bei ihren Experimenten die stark degenerative Wirkung der Essigsäure auf die Leberzellen. Da es sich um Verfütterungsversuche handelt, ist eine indirekte Wirkung über eine Hämolyse oder intrahepatische Thrombosen nicht auszuschließen. Den Beweis für die direkte, leberzellschädigende Wirkung brachten daher erst Parisot und Harter, indem sie durch Injektionen verdünnter Lösungen in den Choledochus herdförmige Leberzellnekrosen zu erzeugen vermochten. Ihre Kaninchen starben in unterschiedlichen Intervallen von 1—18 Tagen und zeigten neben den bereits nach 24 Stunden deutlichen, ausgedehnten und gut abgegrenzten Parenchymnekrosen eine starke Hyperämie der Venen und intralobulären Capillaren, reichliche Rundzelleninfiltrationen der periportalen Felder, insbesondere um die Gallenwege und kleinen Venen, Veränderungen am Gallengangsepithel sowie ein deutliches, frisches, sklerosierendes Bindegewebe in den periportalen Räumen mit Einstrahlung in die interlobulären Spalten nach Art einer eindeutigen Lebercirrhose. Die Milz war stark vergrößert, blutgestaut und pigmentbeladen, ihre Follikel zeigten zentrale Nekrosen.

Die Experimente mit Essigsäure erfolgten fast ausschließlich an Kaninchen und erzielten nicht immer übereinstimmende Resultate. Sie wurden zumeist durch Verfütterung vorgenommen. Die Ergebnisse sind auf den Menschen nur mit Vorbehalt anwendbar, da sich große Schwierigkeiten aus den tiefgreifenden Unterschieden im intermediären Stoffwechsel ergeben. Ein wichtiger Weg aber, Injektionen in die Pfortader, wie sie mit anderen toxischen Substanzen so häufig durchgeführt wurden, ist leider unbeschritten geblieben. Hier vermag unsere Beobachtung ergänzend einzuspringen, da in unserem Falle eine massive Einschwemmung von Essigsäure über die Pfortader in die Leber mit Bestimmtheit anzunehmen ist. Dabei sind wir uns im klaren, daß unsere Beobachtung nur einen einzeln herausgegriffenen Punkt im Ablauf des Geschehens nach Essigsäureeinwirkung auf die Leber darstellt, wenn auch zu einem Zeitpunkt, an welchem besonders interessante Veränderungen in Gestalt der ausklingenden Degeneration und der beginnenden Regeneration zu erwarten sind. Der Beginn einer regenerativen Reaktion der Leber nach toxischer Schädigung wird im allgemeinen um den 1. bis 3. Tag, ihr Höhepunkt um den 6. bis 14. Tag angenommen. Wir können die Ergebnisse, die Parisot und Harter auf dem Weg über Injektionen in den Choledochus erzielten, zum Teil bestätigen. Fassen wir unsere Befunde zusammen, so ergibt sich dabei folgendes.

Die Wirkung der Essigsäure auf das Lebergewebe ist eine abgestufte und dreifache:

1. Eine primäre als direkte Schädigung des Parenchyms,
2. eine sekundäre als andauernde schädigende Wirkung durch Thrombose,
3. eine Schädigung auch der mesenchymalen Anteile des Lebergewebes und damit eine Reizung zur Wucherung des Bindegewebes und zur Narbenbildung.

Als Zeichen der *Gewebsschädigung* finden sich in umschriebenen, infarktähnlichen Bezirken („toxischer Infarkt" Camerons) fleckige und unregelmäßige Nekrosen sowie ausgedehnte degenerativ-dystrophische Veränderungen, vorzüglich der Leberepithelien. Dabei sind die einzelnen Leberläppchen von der Peripherie aus oft scharf sektorartig in Gestalt nicht regeneratorischer Pseudolobuli ergriffen, so daß ein schneller Ablauf der Schädigung bei enger räumlicher Begrenzung der schädigenden Noxe angenommen werden muß; allein durch Thrombosierungen der Pfortaderäste sind solche tiefgreifenden und scharf umschriebenen, die Läppchen zerteilenden Schädigungen nicht vorstellbar, insbesondere, da aus der Art der Thrombosen deren allmähliche Entstehung über eine Stase anzunehmen ist. Wir möchten den Thrombosen der Pfortaderverzweigungen vielmehr im ersten Stadium nur die Rolle einer nachhinkenden Markierung des Weges zusprechen, den die Essigsäure bei ihrer Einschwemmung genommen haben muß. Entsprechend dem Grade der Gewebsschädigung lassen sich alle Übergänge zwischen vollständigem homogen-scholligem Zerfall von Leberepithelien und Atrophien von Leberzellbalken mit Kollaps von Läppchen oder Läppchenteilen und vikariierender Erweiterung der intralobulären Capillaren nachweisen. Sicherlich spielt daneben auch eine direkte Schädigung der Capillarwandungen mit primärer Ektasie und sekundärer Druckatrophie von Leberzellbalken eine Rolle. Verfettungen der angrenzenden Leberzellen sind nicht nur als Folge der Giftwirkung, sondern auch als Folge von Veränderungen im Fettstoffwechsel auf Grund der ausgedehnten Störungen zu deuten.

Als Zeichen der *Regeneration* finden sich zahlreiche, vorwiegend amitotische Zellteilungen von Leberepithelien mit Entwicklung von Riesenzellen und kleinknotigen Hyperplasien, eine Vermehrung von Gitterfasern im Sinne einer inter- und intralobulären Kollapsfibrose, sowie sehr zahlreiche Gallengangswucherungen. Nach Beobachtungen von Manwaring und Clara setzt die Wucherung der Gallengänge und des interlobulären Bindegewebes gleichzeitig mit den proliferativen Vorgängen im Parenchym ein. Herxheimer und Thölldte wiesen auf, daß die Wucherungen der Gallengänge denen des periportalen Bindegewebes durchweg parallel gehen, während umgekehrt „Pseudotubuli" vorwiegend bei akuten Atrophien der Leber zu finden sind. Auch wir finden mehr „Pseudotubuli" als typische Gallengänge, sehen uns jedoch außerstande, zwischen beiden eine scharfe Trennungslinie zu ziehen. Vielmehr lassen sich alle Übergangsformen zwischen typischen Gallengängen und „Pseudotubuli" aufzeigen und sind hier besonders schön und reichlich. Der Gestaltwechsel der „Pseudotubuli" läßt eine gewisse Regelmäßigkeit derart erkennen, daß mit zunehmender Entfernung von den typischen Gallengängen die Epithelien flacher kubisch bis

spindelig, die Kerne ovaler und klarer werden, das Lumen gestaltet sich kleiner und unregelmäßiger und kommt dabei oft exzentrisch zu liegen oder verschwindet ganz. Beachtet man dabei, daß die „Pseudotubuli" besonders dicht vor den peripheren Ausläufern der Leberzellbalken gelegen sind, ja zuweilen sich zwischen diese einschieben, so wird offenbar, wie stark das Bestreben der Gallenwege ist, die Verbindung mit den funktionstüchtig gebliebenen oder wieder gewordenen Leberzellen wiederherzustellen. Der Reichtum der „Pseudotubuli"-Wucherungen muß dabei im Sinne einer Überschußproduktion gewertet werden.

Es erscheint aber unwahrscheinlich, daß die Wucherungen einzig von den interlobulären Gallengängen ihren Ausgang nehmen; vielmehr ist anzunehmen, daß die interlobulären und perilobulären (septalen) Gallengänge sowie deren Zwischenstücke zu den Gallencapillaren zugleich wuchern, wobei der Hauptanteil wohl den Zwischenstücken zukommt. Für die Deutung der „Pseudotubuli" als verschonte und liegengebliebene, meist atrophische Leberzellbalken (Herxheimer und Thölldte) oder als aus Druckatrophien von Leberepithelien infolge Gitterfaserneubildung der intraacinären Capillaren entstanden (Coronini) finden wir keinerlei Anhalt, obwohl unsere Schnitte „Pseudotubuli" und ausgedehnte Parenchymatrophien in reichlichem Maße nebeneinander zeigen. Wir finden zwar „Pseudotubuli" im Bereich von kollabierten Leberzellbalken, aber stets nur in der Peripherie von Leberläppchen, nirgendwo aber im zentralen oder intermediären Läppchenbereich; wir finden zwar Umwandlungen atrophierter Leberzellbalken zu ringförmigen, mit einer Lichtung versehenen Gebilden, doch sind diese in Zellanordnung und Kernstellung von Gallengangswucherungen deutlich verschieden. Vielleicht sind in unserem Fall aber die Veränderungen noch zu jung, um eine genügende intralobuläre Gitterfaserwucherung im Sinne Coroninis zu ermöglichen.

Atrophierte oder regenerierte Leberzellen und „Pseudotubulus"-Epithelien lassen sich im allgemeinen morphologisch auf Grund der besonderen Kern- und Plasmastrukturen und -Färbeeigenschaften eindeutig gegeneinander trennen. Der Plasmaleib der Gallengangsepithelien ist kleiner, weniger eosinophil, heller und feiner strukturiert als der der Leberepithelien, meist kubisch bis spindelig und unterscheidet sich bei Azan durch seine mehr blaßgraue Farbe von dem mehr violett-roten und grob gekörnten Plasma der Leberepithelien. Während die angrenzenden Leberzellen tropfige Fetteinlagerungen häufig enthalten, sind die Epithelien der Gallenwegswucherungen durchweg frei davon, auch zeigen sie nirgends Lipofuscin; ihre Kerne sind deutlich längsoval, glasklar, durchsichtig, chromatinarm und mit mehreren, aber nur spärlichen Kernkörperchen versehen; das zarte und gleichmäßige Chromatinnetzwerk verdichtet sich in der Kernperipherie zu einem scharf gezeichneten Rand. Demgegenüber sind die Kerne der Leberzellen meist rund, undurchsichtiger und zeigen andersartige Chromatinstrukturen.

Wir glauben daher, uns der Meinung Hammars anschließen zu können, wenn er sagt, daß Leberzellen und Gallengänge sich bei der

Ersatzbildung entsprechend den fetalen Bildungsvorgängen getrennt vermehren, können ihm jedoch nicht folgen, wenn er betont, daß alle Übergangsformen zwischen Leberzellen und Gallengangsepithelien vollständig fehlen. Gereizte, zunächst atrophierte und dann regenerierte Leberzellen der äußersten peripheren Läppchenzone lassen sich an ganz vereinzelten Stellen von breit anliegenden „Pseudotubulus"-Epithelien nur noch schwer unterscheiden und ähneln sich weitgehend. Wir sind jedoch nicht imstande, aus diesen, im Verhältnis zur Menge der „Pseudotubuli" sehr seltenen Stellen eine Entstehung der „Pseudotubuli" aus Leberzellen herzuleiten. Es dürfte sich vielmehr um ein gleichzeitiges und gemeinsames Wuchern von Leberzellen und Zwischenstücken der perilobulären, septalen Gallengänge handeln. Anhaltspunkte, die auf eine Umwandlung von Leberzellen zu „Pseudotubuli" oder umgekehrt deuten lassen, *sind nirgends auffindbar.*

Besonders deutlich sind die Zeichen der *Resorption* des zerstörten Gewebes in Gestalt einer ausgedehnten resorptiven Entzündung. Dichte rundzellige Infiltrationen aus Lymphocyten, Plasmazellen und gelapptkernigen Leukocyten umgeben gürtelartig die Parenchymnekrosen und finden sich noch weit ab in den verbreiterten periportalen Feldern zwischen gesunden Leberläppchen. Die erweiterten Capillaren der den Nekrosen benachbarten Leberläppchen enthalten reichlich kernhaltige Blutelemente. Die Kupfferschen Sternzellen innerhalb der Parenchymnekrosen sowie die gewucherten Bindegewebszellen und intralobulären Capillarendothelien in deren Umgebung tragen reichlich phagocytiertes Zerfallspigment, letztere auch eisenhaltiges Pigment aus capillären Blutungen. In den Gallenwegen liegen neben Pigment auch Leukocyten als Ausdruck einer Ausscheidungscholangitis.

Als Zeichen einer direkten *Reizung des Mesenchyms* finden sich eine Vermehrung, Schwellung, Abrundung und Ablösung der Capillarendothelien innerhalb der Parenchymnekrosen wie noch weit inmitten der benachbarten erhaltenen Leberläppchen im Sinne einer ausgeprägten, intralobulären Retikuloendothelreaktion, sowie eine starke Wucherung der Bindegewebszellen im Bereich der angrenzenden periportalen Felder, vornehmlich in Gestalt von Fibroblasten. Letztere haben mit der Neubildung kollagener Fibrillen begonnen und stellenweise bereits zu Druckschädigungen an benachbarten Leberzellbalken geführt. Innerhalb der geschädigten Parenchymteile ist eine leichte Verdickung und kollagene Imprägnierung des Gitterfasergerüstes im Sinne einer feinfaserigen Sklerosierung erkennbar. Wie weit hierbei eine Abhängigkeit zu faserbildenden Zellen besteht, läßt sich wegen der gleichzeitigen starken Endothelreizung nicht entscheiden.

Damit berühren wir die Frage der Entstehung von Lebercirrhose nach Schädigung durch Essigsäure. Eine Cirrhose nach chronischer

Leberatrophie ist frühestens nach 4 Monaten zu erwarten (Rössle) und insofern läßt unser Fall, bei dem der Beginn der Schädigung 8 Tage vor dem Tode zurückliegt, wenig erhoffen. Wir glauben indessen, aus der Besonderheit gewisser Veränderungen einiges zur Genese der Lebercirrhose beitragen zu können.

Zunächst sei betont, daß es sich in unserem Fall um herdförmige, infarktähnliche Schädigungen der Leber gehandelt hat. Partielle Cirrhosen sind jedoch verschiedentlich beschrieben worden, so unter anderem von Rössle mit typischer Laennecscher Höckerung und einer Ausdehnung gegen die Tiefe von etwa 1 cm als wahrscheinliche Folge gröberer Gefäßverlegung. Wenn Kretz, Joannovicz und Fischler eine in Schüben wiederholte Parenchymschädigung als das Primäre bei der Lebercirrhose ansprechen, so steht demgegenüber die Tatsache, daß auch weitgehende Parenchymausfälle noch vollständig regeneriert werden können (Jaffé), ohne daß es zur Cirrhosierung kommt. Zur Lebercirrhose bedarf es einer gleichzeitig gegen Leberepithel und Mesenchym gerichteten Giftwirkung, neben den Parenchymverlusten noch einer schleichenden Entzündung. In unserem Fall wird die starke resorptive Entzündung ergänzt durch eine direkte Reizung des Mesenchyms mit Wucherungen der Capillarendothelien und periportalen Bindegewebszellen, sowie durch tiefgreifende Störungen der Kreislaufverhältnisse im Bereich der intra- und perilobulären Capillaren, besonders begünstigt durch das zerstreut-fleckige und die Leberläppchen-Zerteilende der Parenchymnekrosen. Nach Rössle stellt die Schädigung des Gefäßnetzes die Einleitung des cirrhotischen Prozesses dar, und bindegewebige Wucherung und Parenchymentartung sind deren Folgen. Jaffé sieht ein wesentliches Moment im vermehrten Blutzerfall, doch spricht Rössle den rein erythrocytolytischen Giften jede cirrhogene Wirkung ab. Erst die Mitbeschädigung der Capillarwände und Leberzellen macht die Blutgifte zu Auslösern narbenbildender Hepatitis, und in diesem Sinne sind auch die Versuche mit Hämoglobinlösungen (Jaffé), hämolytischen Giften (Paerce) und Fettsäuren (Boix) zu verstehen. Um eine echte partielle Cirrhose hervorzurufen, müßte die schädigende Noxe jedoch einige Zeit ständig und wiederholt einwirken. Diese andauernde Wirkung wird für unseren Fall durch die ausgedehnten Thrombosierungen der feinen Pfortaderverzweigungen gesichert.

Zum Bilde der Lebercirrhose gehört schließlich eine ausgedehnte, infolge mangelhafter Wiederherstellung des Gewebes entstandene Narbenbildung. Als deren erste Zeichen finden wir eine zarte pericapilläre sowie eine stärkere periportale Bindegewebswucherung im Bereich der geschädigten Leberbezirke. Stellenweise sind die intralobulären Capillaren verödet und haben sich in feine bindegewebige

Stränge umgewandelt; als die Lieferanten des Bindegewebes müssen hierbei letzten Endes die Endothelien angesprochen werden. Hiermit bestätigten wir die Befunde von Pearce, der Neubildung von intercellulären Fibrillen bereits am 5. Tag und von Blutgefäßen am 11. Tag nach Injektionen von hämolytischen Immunseren beobachtete.

Infolge der narbigen Schrumpfungen zeigt die Leberkapsel dort, wo die Gewebsnekrosen die Oberfläche berühren, kleine trichterförmige Einziehungen. Ausgedehnte Gallengangswucherungen, sowie die nichtregeneratorischen „Pseudolobuli" vervollständigen das Bild zu dem einer im ersten Beginn stehenden Cirrhosierung.

Auf Grund der histologischen Bilder sind wir also berechtigt anzunehmen, daß die Essigsäure bei ihrer Einschwemmung durch die Pfortader eine Verwundung des Lebergewebes als Ganzes setzt. Sie ist demnach in die große Gruppe der Cirrhose auslösenden Gifte einzureihen.

Zusammenfassung.

Es wird über eine akute perorale Vergiftung durch hochkonzentrierte Essigsäure berichtet, an der der Patient am 8. Tage starb; die Autopsie ergab neben hochgradigen Verätzungen des Verdauungstraktes ausgedehnte Thrombosierungen der Magen-, Netz- und Milzvenen, sowie peripherer Pfortaderverzweigungen mit landkartenartigen anämischen Infarktnekrosen in Leber und Milz, die als direkte Folgen einer Überschwemmung des Blutes mit Essigsäure aufgefaßt werden.

Die Schädigungen der Leber ähneln am ehesten denen bei der Eklampsie und sind durch tiefgreifende, mit Thrombosierungen einhergehende Kreislaufstörungen im Bereich der Pfortaderäste und der intralobulären Capillaren, durch Nekrosen und Atrophien des Parenchyms, sowie durch eine Reizung des Mesenchyms mit starker Retikuloendothelreaktion und ausgedehnten rundzelligen Infiltrationen gekennzeichnet.

Hierauf antwortet das Lebergewebe frühzeitig mit Regenerationen von Leberepithelien wie Wucherungen des periportalen und intralobulären Bindegewebes und der Gallengänge.

Die Herkunft der zahlreich ausgebildeten sog. Pseudotubuli wird zu deuten versucht.

Auf Grund der genauen Kenntnis über die Dauer der Gifteinwirkung ergeben sich neue Anhaltspunkte für die zeitliche Bemessung des Beginns von Leberregeneration und cirrhotischer Narbenbildung.

Die Essigsäure gehört wegen ihrer das Lebergewebe gleichzeitig schädigenden und reizenden Wirkung zur Gruppe der Cirrhose auslösenden Gifte.

Literatur.

d'Amato, L.: Virchows Arch. 187, 435 (1907). — Bergmann, G. v.: Klin. Wschr. 1927 I, 776. — Boix, E. Ph.: Thèse des Paris. 1894. — Boruttau: Berl. klin. Wschr. 1920 II, 1174. — Cameron, G. R., W. A. E. Karunaratne and J. C. Thomas: J. Path. a. Bacter. 44, 297 (1937). — Coronini, C.: Wiener med. Wschr. 1935 I, 231. — Doljanski, L. u. Fr. Roulet: Virchows Arch. 292, 256 (1934). — Esau: Ärztl. Sachverst.ztg. 1913. — Fischler, F.: Dtsch. Arch. klin. Med. 93, 427 (1908). — Franz: Friedreichs Bl. gerichtl. Med. 60 (1909); 61 (1910). — Gerhartz, H.: Fühners Slg Vergift.fällen I, 27. — Hammar, J. A.: Z. mikrosk.-anat. Forsch. 5, 59 (1926). — Heine, C.: Virchows Arch. 41, 24 (1867). — Herxheimer, G. u. Thölldte M.: In Henke-Lubarschs Handbuch der speziellen pathologischen Anatomie, Bd. V/1. 1930. — Jaffé, R. H.: Frankf. Z. Path. 24, 241 (1920). — Joannovicz, G.: Wiener klin. Wschr. 1904, 757. — de Josselin de Jong: Mitt. Grenzgeb. Med. u. Chir. 24, 160 (1911). — Jost, H.: In Handbuch der normalen und pathologischen Physiologie, Bd. V. 1928. — Kretz, R.: Virchows Arch. 222, 30 (1916). — Loeb u. Meyers: Virchows Arch. 210, 78 (1910). — Marx, M.: Prag. med. Wschr. 1914 II, Nr 29 u. 30. — Meyer, E.: In Handbuch der normalen und pathologischen Physiologie, Bd. VI/1. 1928. — Parisot, J. u. A. Harter: C. r. Soc. Biol. 1907, 530. — Pearce, R. M.: J. exper. Med. (Am.) 8, 64 (1906). — Petri, I.: In Henke-Lubarschs Handbuch der speziellen pathologischen Anatomie, Bd. X. 1930. — Pfuhl, W.: In v. Möllendorf.s Handbuch der mikroskopischen Anatomie des Menschen, Bd. V/2. 1932. — Raestrup, G.: Untersuchungen über die Widerstandsfähigkeit der Leber gegen Gifte. Leipzig 1927. — Reddingius, R. A.: Verh. dtsch. path. Ges. 1912. — Rössle, R.: In Henke-Lubarschs Handbuch der speziellen pathologischen Anatomie, Bd. V/1. 1930. — Schäffer, E.: Ärztl. Sachverst.ztg. 1902, 15. — Schibkow, A.: Vjschr. gericht. u. öff. Med. 55, 187 (1918). — Schmorl, G.: Verhdl. dtsch. path. Ges. 1902. — Schulz, O. u. L. R. Müller: Dtsch. Arch. klin. Med. 76, 544 (1903). — Silbermann, O.: Z. klin. Med. 1886, 459. — Vinzenzo: Riforma med. 1904, Nr 4.

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Biosynthesis of fatty acids from [1-^{14}C]acetate in the perfused rat heart

Fatty acid biosynthesis in the heart has been ascribed chiefly to the mitochondria[1,2] in contrast to organs such as the liver, adipose tissue and mammary gland in which the fatty acid synthesizing enzymes are mainly in the cell cytoplasm. Synthesis in the mitochondria is generally by the process of elongation contrasted to the *de novo* synthesis which usually occurs in cell cytoplasm. WHEREAT, HULL AND ORISHIMO[3] reported that rabbit heart mitochondria were very active in fatty acid synthesis and, when incubated with [1-^{14}C]acetate, yielded shorter (12–16 carbons) fatty acids synthesized *de novo* and longer-chain fatty acids formed by the elongation of preformed acyl units with the added labeled acetate. HOWARD[4] concluded that *de novo* synthesis occurred in the outer membrane of the mitochondrion, while others[5,6,8] have suggested that little or no *de novo* synthesis takes place.

The question arises as to the types of fatty acids synthesized and the mode of synthesis occurring in the intact heart *in vivo*. In rat heart perfused with [1-^{14}C]acetate, we have found the major portion of the incorporated ^{14}C in saturated and monoenoic acids of 14-, 16- and 18-carbon chain lengths. Palmitic acid was synthesized by a *de novo* pathway, while stearic acid was formed by a pathway involving elongation.

Hearts taken from adult female Sprague-Dawley rats weighing between 250 and 300 g and maintained on Purina rat chow were perfused in an apparatus similar to that described by MORGAN *et al.*[7] for coronary perfusion in a recirculating system. The gas mixture used was O_2–CO_2 (95:5, v/v), and the perfusion medium was Krebs–Henseleit bicarbonate buffer (pH 7.4) maintained at 37.5°. The heart was removed from the animals under nembutal anesthesia, transferred to the perfusion chamber and washed for 3 min with medium before starting on the recirculation system with 25 ml of perfusion medium containing the labeled substrate. Both the washing medium and the perfusing medium contained glucose at a concentration of 5 mM. The labeled fatty acid precursor was 5 μC of [1-^{14}C]acetate at a concentration of 5 mM. Perfusion was carried on for 2 h. If the heart beat, which generally was 200–220 per min, dropped below 160, the preparation was discarded.

Fatty acids were isolated by extraction after hydrolysis with ethanolic KOH (containing 0.1 ml of 0.5% solution of hydroquinone as antioxidant) and subsequent acidification. Radioactivity of total fatty acids was determined in a liquid scintillation spectrometer. Radioactivity of individual fatty acids was determined by gas–liquid radiochromatography of the methyl esters using a heated continuous-flow proportional detector (Nuclear Chicago Model 4998). Diethylene glycol succinate polyester (12%) on 110/120 mesh Anakrom ABS was used for column packing. For isolation of pure palmitic and stearic acids for degradation, a preparative column packed with diethylene glycol succinate polyester (20%) on Chromosorb W (100/140) mesh was used, and the samples were collected on siliconized glass wool packed in long tubes which were heated at the end connected to the exit port of the mass detector. Collected fractions were subjected to final purification by column chromatography on silicic acid $AgNO_3$ (25% $AgNO_3$) using the elution schedule of DE VRIES[9]. For chemical degradations, the method of DAUBEN *et al.*[9] was used. Total lipids extracted

with Folch mixture (chloroform–methanol) were fractionated into lipid classes by thin-layer chromatography using silicic acid. The fractions were eluted from the plates by the method of BIEZENSKI[10]. Sodium [1-^{14}C]acetate was obtained from New England Nuclear Corporation, column packings for gas chromatography from Analabs, and standards for gas chromatography and silicic acid for chromatography from Applied Science Laboratories.

Incorporation of [1-^{14}C]acetate into fatty acids determined in perfused hearts of 10 rats averaged 0.24 ± 0.05% (S.E.) of the added substrate. Results of gas–liquid radiochromatographic analyses of these ten samples are given in Table I in the form of average values ± S.E. Very little ^{14}C activity was present in fatty acids of chain length shorter than 12 carbons. The major portion of the incorporated radioactivity

TABLE I

DISTRIBUTION OF ^{14}C FROM [1-^{14}C]ACETATE IN FATTY ACIDS SYNTHESIZED BY PERFUSED HEART

Fatty acid	*% of recovered ^{14}C (average of 10 samples ± S.E.)*
$<C_{10:0}$	0.7 ± 0.1
$C_{12:0, 12:1}$	2.8 ± 0.0 (includes trace of $C_{12:1}$)
$C_{14:0, 14:1}$	9.1 ± 1.3 (includes approx. 0.5% 14:1)
$C_{16:0, 16:1}$	48.1 ± 4.3 (includes approx. 2.0% 16:1)
$C_{18:0, 18:1}$	26.6 ± 3.6 (includes approx. 9.0% 18:1)
$C_{20:2}$	2.2 ± 0.6
$C_{20:4}$	3.4 ± 1.2
$>C_{20:4}$	1.8 ± 0.9

was in the 14-, 16- and 18-carbon fatty acids (saturated and monoenoic). Small amounts of radioactivity were present in fractions identified by retention time as 20:2 and 20:4, in other compounds of higher degree of unsaturation than 4 double bonds (possibly 20:5) and also of 22-carbon chain length. Confirmation of the latter was obtained by hydrogenation of an aliquot of one of these samples and determination of the ^{14}C activity of the resulting fatty acids by gas–liquid radiochromatography.

The distribution of the radioactivity in the chain was determined by decarboxylating pure palmitic and stearic acids isolated from pooled samples. Results of these analyses are given in Table II. The average ratio (specific activity carboxyl carbon to specific activity average carbon in the entire chain) resulting from analyses of two different pooled samples of palmitic acid was 2.0, which is the theoretical value expected if this acid had been synthesized *de novo* from [1-^{14}C]acetate. On the other hand, a ratio greater than two, as obtained for stearic acid, indicates that some degree of elongation occurred in the synthesis of this acid.

The ^{14}C activity of fatty acids of various lipid fractions separated from a total lipid extract by thin-layer chromatography was distributed in the following manner: phospholipids 78%, triglycerides 17%, free fatty acids 4% and cholesterol esters 0.5%.

Our results indicate that the perfused rat heart used [^{14}C]acetate for synthesis of palmitic acid by a *de novo* pathway and of stearic acid at least partially by elongation. Results similar to ours were obtained by WHEREAT *et al.*[3] using rabbit heart mitochondria.

There is some disagreement among investigators on whether there is *de novo*

TABLE II

SPECIFIC ACTIVITY OF AVERAGE FATTY ACID CARBON AND OF CARBOXYL CARBON OF PURE PALMITIC AND STEARIC ACIDS ISOLATED FROM POOLED HEART FATTY ACIDS

Fatty acids		*Specific activity (counts/min per mg C)*		*Ratio B/A*
		Average fatty acid carbon (A)	*Carboxyl carbon (B)*	
16:0	Sample 1	30.0	65.0	2.2
	Sample 2	12.1	21.2	1.8
18:0	Sample 1	4.6	42.7	9.3
	Sample 2	0.03	0.5	7.0

synthesis in mitochondria[2,4,11–14]. It has also been suggested by CHRIST AND HÜLSMANN[15] that the mitochondrion is the major intracellular site of fatty acid synthesis in the heart. If this is indeed so and if there is little or no *de novo* synthesis in mitochondria, then results of our experiments in the intact perfused heart do not agree with those obtained by others with isolated particles. Although the results of studies reported in this paper do not define in what part of the cell palmitic and stearic acids were synthesized, the data clearly indicate that *de novo* synthesis and elongation of fatty acids are both used by the perfused rat heart and presumably, therefore, by the intact rat heart *in vivo*.

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1 W. C. HÜLSMANN, *Biochim. Biophys. Acta*, 58 (1962) 417.
2 J. V. DAHLEN AND J. W. PORTER, *Arch. Biochem. Biophys.*, 127 (1968) 207.
3 A. F. WOERREAT, F. E. HULL, M. W. ORISHIMO AND J. L. FABINOWITZ, *J. Biol. Chem.*, 242 (1967) 4013.
4 C. F. HOWARD, JR., *Biochim. Biophys. Acta*, 164 (1968) 448.
5 E. J. V. J. CHRIST, *Biochim. Biophys. Acta*, 152 (1968) 50.
6 E. QUACIARIELLO, C. LANDRISCINA AND P. CORATELLI, *Biochim. Biophys. Acta*, 164 (1968) 12.
7 H. E. MORGAN, M. J. HENDERSON, D. M. REGEN AND C. R. PARK, *J. Biol. Chem.*, 236 (1961) 253.
8 B. DE VRIES, *J. Am. Oil Chemists' Soc.*, 40 (1963) 184.
9 W. C. DAUBEN, E. HOERGER AND J. W. PETERSEN, *J. Am. Chem. Soc.*, 75 (1953) 2317.
10 J. J. BIEZUNSKI, *Federation Proc.*, 23 (1964) 503.
11 E. M. WEE-PEETERS, *Biochim. Biophys. Acta*, 176 (1969) 453.
12 W. R. HAPLAN AND S. J. WAKIL, *J. Biol. Chem.*, 238 (1963) 3216.
13 E. J. BARRON, *Biochim. Biophys. Acta*, 116 (1966) 425.
14 W. C. HÜLSMANN AND D. S. DOW, *Biochim. Biophys. Acta*, 84 (1964) 486.
15 E. J. CHRIST AND W. C. HÜLSMANN, *Biochim. Biophys. Acta*, 60 (1962) 72.

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